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THE UNIVERSITY OF ALBERTA

COMPARATIVE STUDIES OF SEVEN *GIARDIA* ISOLATES FROM VARIOUS ANIMAL HOSTS IN SOUTHERN ALBERTA

BY

AKIKO UJI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Comparative studies of seven Giardia isolates from various animal hosts in southern Alberta submitted by Akiko Uji in partial fulfillment of the requirements for the degree of Master of Science in Microbiology.

Supervisor

Date: 8 August 1989

ABSTRACT

Seven Giardia isolates from various animal hosts in southern Alberta and one Giardia human isolate of Afghanistan origin, WB, were compared in their morphology, DNA fragment patterns, isozymes, antigens, and intracellular pH. The seven isolates were obtained from two humans, two beavers, one muskrat, one sheep and one dog. The bacterial contamination of the original cultures was eliminated by treating trophozoites with penicillin, gentamicin and ticarcillin. All isolates were morphologically indistinguishable from G. duodenalis, and their sizes ranged from 13.6 to 17.6 μm in length and from 7.2 to 8.2 μm in width. Ethidium bromide staining of DNA restricted with BamHI, HindIII or PstI did not show differences in DNA fragment patterns. Hybridization of these restricted DNAs with ^{32}P-labeled whole Giardia DNA probes, prepared from two human isolates and one dog isolate, did not show differences, either. Electrophoretic mobility of glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), malate dehydrogenase and glucose phosphate isomerase from 8 isolates were identical when analyzed by PAGE. Apparent Michaelis constants of G6PD from 8 isolates were 194 to 346 μM, and those of ME were 150 to 276 μM. Monoclonal antibody raised against WB agglutinated trophozoites of all isolates without cytotoxicity, and reacted similarly with all isolates in the immunofluorescence assay. This antibody immunoprecipitated a surface antigen of WB trophozoite, 99 kDa in size, which was not detected on a Western blot of WB antigens prepared by SDS-PAGE. Intracellular pH of 8 isolates ranged from 6.38 to 6.74. These results indicate that Giardia isolates obtained from different

animal hosts in southern Alberta are very similar to one another. They showed very low genetic diversity in DNA and isozymes, and shared a common surface antigen of 99kDa with an epitope susceptible to SDS. The differences in their morphology, intracellular pH, and apparent Michaelis constants of two isozymes were low. Therefore, although they are also similar to WB of Afghanistan origin, it is likely that Giardia isolates originating in southern Alberta are closely related to each other regardless of their original host species, and cross-transmission of Giardia among various animals in this area may exist.

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LIST OF ABBREVIATIONS

BCECF.....bis (carboxy-ethyl) carboxy fluorescein acetoxymethyl ester

bp.....base pairs

BSA.....bovine serum albumin

CPM.....counts per minute

DMSO.....dimethyl sulfoxide

DNA.....deoxyribonucleic acid

DNase.....deoxyribonuclease

EDTA.....ethylenediamine tetraacetic acid

ELISA.....a double antibody enzyme-linked immunosorbent assay

FCS.....heat inactivated fetal calf serum

FSB.....final sample buffer

x g.....times gravitational force

GPI.....glucose phosphate isomerase (E.C. 5.3.1.9)

G6PD.....glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49)

kb.....kilo bases

kDa.....kilo dalton

Km.....Michaelis constant

L:W.....length : width

MDH.....malate dehydrogenase NAD^+ (E.C. 1.1.1.37)

ME.....malic enzyme (E.C. 1.1.1.40)

MTT.....methyl thiazolyl tetrazolium methyl thiazolyl blue

NADP.....nicotinamide adenine dinucleotide phosphate

OA-TSA.....2% ovalbumin in TSA

PAGE.....polyacrylamide gel electrophoresis

PA-Sepharose...protein A-Sepharose suspension in RIPA buffer

PBS.....50 mM phosphate-buffered saline (pH 7.2)

PBS-T.....100 mM phosphate-buffered saline (pH 7.0) containing 0.1% Tween-20

PEG.....polyethylene glycol solution

PMS.....phenazine methosulphate

RNA.....ribonucleic acid

RNase.....ribonuclease

RPMI+GE.....RPMI 1640 containing 200 μg/mL gentamicin

SDS.....sodium dodecyl sulfate

SEM.....scanning electron microscopy

tris.....tris(hydroxymethyl)aminomethane

TSA-Y.....trypticase soy agar supplemented with 0.5% yeast extract

1. Introduction

Giardia duodenalis is a flagellated protozoan living in the small intestine of a wide range of mammals (Nash et al. 1983) such as mice, dogs, beavers, rabbits and humans (Kudo 1966). Human infection is known as giardiasis or giardiosis (Marquardt & Demaree 1985).

Its life cycle consists of two stages (Larsh 1980); parasitizing trophozoites (Marquardt & Demaree 1985) and infective cysts (Feely et al. 1984). Trophozoites are pyriform to ellipsoid, from 12 to 15 μm in length and from 6 to 8 μm in width (Feely et al. 1984), with two nuclei, two axostyle and eight flagella in four pairs (Kudo 1966) (Fig. 1). The anterior end is broadly rounded and the posterior end is drawn out (Kudo 1966). The dorsal side is convex and the ventral side is concave or flat with a ventral disc in the anterior half (Kudo 1966). Cysts are oval to ellipsoid from 8 to 14 μm in length and from 6 to 10 μm in width, with two or four nuclei, axostyles, and fibrils (Kudo 1966).

Infection occurs when cysts are swallowed by animal hosts with contaminated food or water (Olsen 1974). The swallowed cysts excyst in the duodenum (Olsen 1974). One cyst produces one mature tetranucleate flagellate which quickly undergoes cytoplasmic division, forming two binucleate daughter trophozoites (Olsen 1974). In the small intestine, the trophozoites reproduce by longitudinal binary fission with no sexual stages (Marquardt & Demaree 1985). This multiplication involves nuclei, ventral disc and other parts, and separates the parasite into two daughter trophozoites (Olsen 1974). Individual trophozoites rest on the surface cells of the intestine, adhering by a ventrolateral flange and a

Fig. 1 has been removed because of the unavailability of copyright permission.

Figure 1 Diagram of G. duodenalis illustrating the morphological details (Ref. Farmer 1980).

ventral disc (Olsen 1974). The trophozoite maintains adhesion by beating ventral flagella strongly which causes a suction pressure in the channel formed by the ventrolateral flange and the ventral disc (Holberton 1974).

After a period of multiplication by binary fission, trophozoites start encystment (Olsen 1974) in the lower small intestine (Marquardt & Demaree 1985). They withdraw the flagella, shorten the body, and secrete a tough and hyaline cyst wall to coat themselves (Olsen 1974). Inside the cyst, each flagellate undergoes a process of maturation to develop four nuclei and two ventral discs (Olsen 1974). These mature cysts, which appear in formed stools, are infective as well as resistant to unfavorable environmental conditions (Olsen 1974) and cause the infection of new hosts (Marquardt & Demaree 1985).

Generally, the infecting site of G. duodenalis is thought to be the upper part of the small intestine (Olsen 1974, Farmer 1980, Larsh 1980, Marquardt & Demaree 1985), where trophozoites attach on the surface of the intestinal mucosa without invading the tissues (Gillon & Ferguson 1984).

The signs and symptoms most often seen in giardiasis are bloating or flatulence, diarrhea, and weight loss in the face of good appetite (Barbour et al. 1976). However, infection can result in a wide range of responses from none at all to a severe, debilitating diarrhea and other symptoms (Wolfe 1984, Marquardt & Demaree 1985).

The specific pathogenic mechanisms of G. duodenalis infecting humans are not yet understood (Wolfe 1984), but direct mechanical injury is the most likely mechanism for the majority of symptoms of giardiasis (Craft 1982). The attachment of trophozoites to the mucosal surface

injures the microvillus epithelium, which leads to a functional disorder in the cell membrane and a fuzzy coat (Hartong et al. 1979). Diarrhea, a major symptom of giardiasis, can be explained by this mechanism (Craft 1982).

Also, this mechanical injury of the microvillus epithelium may be responsible for the malabsorption of materials from the small intestine, which is another symptom often observed in giardiasis (Craft 1982). Rapid migration of less mature columnar cells to the villae surface replacing damaged cells is responsible for reduction in brush border enzymes (Craft 1982). Hartong et al. (1979) studied the functional - structural changes of the small intestinal mucosa of 28 symptomatic patients with giardiasis. They found a significant reduction in brush border enzymes such as lactase and sucrase, along with malabsorption of D-xylose and vitamin B_{12}.

G. duodenalis has worldwide distribution, being more common in children than in adults and in warm than in cold climates (Olsen 1974). Human infection occurs in 200 million persons per year and causes 500,000 cases of diarrhea per year in the world (Warren 1983). The number of diseased patients is small compared with that of infected people because there are many asymptomatic cases. In the U.S.A., giardiasis is considered to be the most common pathogenic intestinal parasite (Wolfe 1975). In several stool surveys, Giardia was demonstrated in 2 to 22% of individuals in healthy populations in North America (Healy 1979). Other surveys indicate about a 7% prevalence in temperate North America, and some populations show as many as 67% of persons to be infected (Marquardt & Demaree 1985). It is also the most frequently reported intestinal parasite in Britain (Knight & Wright

1978). In Canada, there were 4559 reported cases of giardiasis in 1984 and probably there were many more unreported cases (Woo & Paterson 1986).

Human infection occurs not only as sporadic individual infections but also as epidemics (Craun 1984, Marquardt & Demaree 1985). Outbreaks in the community have been traced to contaminated food, water, person-to-person spread in day-care centers, institutions for mentally retarded persons, homosexuals, camps and skiers (Woo & Paterson 1986). Among them, epidemics are usually associated with faulty water delivery systems (Marquardt & Demaree 1985). A classic example of waterborne outbreak occurred in the ski resort in Aspen, Colorado, where old sewer pipes leaked sewage into a well serving a part of the town (Moore et al. 1969). At least 25 persons suffered from clinical infections with G. duodenalis (Moore et al. 1969).

The incidence of giardiasis over any given period in a locality is not well documented (Marquardt & Demaree 1985). However, in Colorado, where giardiasis is a reportable disease, the State Department of Health generally lists about 400 cases per year (Marquardt & Demaree 1985). In the past decade more than 7,000 waterborne cases have occurred in more than 20 areas primarily in mountainous parts of New England, the Pacific Northwest and the Rocky Mountains (Juranck 1979, Craun 1979).

Most of the recent outbreaks resulted from the consumption of untreated surface water or chlorinated but unfiltered water from small municipal water systems or semipublic water systems in recreational areas (Walsh 1983). The water supplies became contaminated from animal or human sources and cysts remained viable in the usual chlorine concentrations in the purification systems (Walsh 1983). The levels of

chlorine usually employed in municipal water treatment plants cannot kill Giardia cysts and, in addition, they can survive in water for up to 3 months (Marquardt & Demaree 1985). When contaminated water was consumed, people became infected despite chlorination, and showed the clinical signs and symptoms of giardiasis (Marquardt & Demaree 1985).

In most instances of waterborne outbreaks, contamination of the water supply seemed not to come from human excreta (Marquardt & Demaree 1985). It seems likely that some animals other than humans are the source of the contamination (Marquardt & Demaree 1985). Dogs, cats and beavers have been implicated but there is not enough evidence to prove that feces of these animals provided the cysts that entered the water supply (Davis & Hibler 1979).

Lewis (1984) studied the prevalence of Giardia infection in dogs during a major outbreak of human giardiasis which occurred in 1983 in Edmonton, Alberta. His study showed that 17% of 99 dogs from the Edmonton Municipal Animal Shelter were infected during the peak of human infection. In contrast, only 3.2% of 218 dogs were infected in the previous year when there was no human outbreaks of giardiasis (Lewis 1984). This result suggests that some Giardia strains can infect both dogs and humans and that the interspecies transmission of Giardia, especially between humans and other animals, may be possible.

To study the possibility of cross transmission of Giardia among different animal hosts, it would be important to identify Giardia species isolated from various animals in a single geographic area. However, there is no valid method to identify Giardia species, because their taxonomic criteria have not yet been defined (Korman et al. 1986).

In the past, Giardia species were defined by the species of their original hosts and the morphology and dimensions of trophozoites (Kudo 1966). For example, isolates from dogs, mice, cats, and oxen were referred to as G. canis, G. muris, G. cati, and G. bovis, respectively (Kudo 1966, Marquardt & Demaree 1985). In this way, nearly 50 species of Giardia have been reported (Friend 1966).

The disadvantages of this taxonomy are that it ignores the possibility that some of the isolates are the same species infecting different animals, and that it allows too many species to be defined. Because of this inconvenience, Filice (1952) suggested broader classification. He proposed 3 Giardia species with the following definitions;

G. duodenalis: Hosts are mammals. Median body is a single or double structure, claw shaped, and located transversely in the mid portion of the cell. Body shape is intermediate to G. muris and G. agilis.

G. muris: Hosts are rodents, birds, and reptiles. Two small median bodies are located in the center of the cell. Body is slightly longer than the width.

G. agilis: Hosts are amphibia. Median bodies are teardrop-shaped and aligned parallel to the longitudinal axis of the cell. Body is long and narrow.

The median body described in these definitions is a structure unique to Giardia (Feely et al. 1984). It is mainly composed of microtubules which can be stained with hematoxylin, and its function is unknown (Feely et al. 1984).

Filice's proposal has been accepted by many authors, and now Giardia isolates obtained from mammals are usually referred to in the literature as G. duodenalis, although human isolates are still often called G. lamblia (Marquardt & Demaree 1985).

However, it is not known how many valid species are included in the isolates designated to be G. duodenalis. Actually, several authors demonstrated heterogeneity of G. duodenalis isolates. The differences have been shown in many aspects.

Erlandsen and Bemrick (1987) reported that G. duodenalis isolates from parakeets are morphologically different from other G. duodenalis. They isolated Giardia trophozoites from the small intestine of parakeets and examined these with light and scanning electron microscopy. These trophozoites, like other Giardia species, were flattened dorso-ventrally and possessed 8 flagella and an adhesive disc on the ventral surface. The presence of a claw hammer-shaped median body suggested classification of these trophozoites as G. duodenalis. However, unlike any known member of G. duodenalis, the Giardia trophozoites from parakeets were morphologically distinct in that they lacked the ventrolateral flange. This distinct morphology clearly indicated that trophozoites from parakeets should be considered as a separate species, G. psittaci.

Grant and Woo (1978a) studied differences in host specificities between G. duodenalis isolates. They collected Giardia from various small rodents livetrapped at 6 locations in Southern Ontario. From the general morphology and original host species, they identified 3 species, G. simoni, G. peromysci and G. microti. These isolates showed no remarkable morphological difference among them, and could be defined as G. duodenalis according to the Filice's criteria. However, the results of their cross-transmission experiments showed that these isolates were different species having different host specificities. In their experiments, cysts from original hosts were inoculated into 5 different species of recipient animals; laboratory rats, laboratory mice, golden hamsters, meadow voles and deer mice. Feces of these animals were examined periodically for 28 days to detect infection. The results showed that each isolate had a very high host specificity. G. simoni isolated from a laboratory rat infected only laboratory rats. G. peromysci isolated from a deer mouse infected only deer mice. Although G. microti infected meadow voles and golden hamsters, meadow vole was its original host. This result indicates that the 3 classifications of Giardia species suggested by Filice (1952) is rather a rough classification, and Giardia isolates classified as G. duodenalis can be different species.

Nash et al. (1985) showed differences in DNA banding patterns between G. duodenalis isolates. DNA of 11 human and four animal isolates (two beavers, one cat, and one guinea pig) of Giardia were compared. Two major groups could be distinguished with ethidium bromide staining of DNA from 8 isolates restricted with endonuclease. Southern blot analysis using recombinant plasmids containing Giardia DNA as

probes could distinguish nine different patterns among 15 isolates studied.

Difference in zymodemes among various G. duodenalis isolates were demonstrated by several authors by electrophoresis of isozymes. Betram et al. (1983) analyzed 6 different enzymes from 5 Giardia isolates by starch and polyacrylamide gel electrophoresis (PAGE). The isolates were obtained from a human, a cat, and a guinea pig. The enzymes analyzed were malate dehydrogenase NAD^+ (MDH; E.C. 1.1.1.37), malic enzyme (ME; E.C. 1.1.1.40), glucose-6-phosphate dehydrogenase (G6PD; E.C. 1.1.1.49), 2-glycerophosphate dehydrogenase (E.C. 1.1.1.8), hexokinase (HK; E.C. 2.7.1.1) and 6-phosphogluconate dehydrogenase (E.C.1.1.1.44). Comparing the zymodemes, they could divide 5 isolates into 3 groups and they found 2 human isolates from Bethesda and England were identical with a cat isolate from Portland.

Korman et al. (1986) analyzed 5 human isolates; Giardia WB isolate and another 4 isolates from Jerusalem. WB is a human isolate of Afghanistan origin. Five enzymes, ME, glucose phosphate isomerase (GPI; E.C. 5.3.1.9), alanine aminotransferase (E.C. 2.6.1.2), aspartate aminotransferase (E.C. 2.6.1.1), and nucleoside hydrolase (E.C. 3.2.2.2) were analyzed by starch gel electrophoresis. Only 2 isolates, both from Jerusalem, were identical. They showed significant heterogeneity in G. duodenalis isolates, both from widely separated areas and within a single area.

Baveja et al. (1986) analyzed 4 human isolates, one from Portland and 3 from New Delhi. GPI, ME, phosphoglucomutase (E.C. 2.7.5.1) and HK were analyzed by starch gel electrophoresis. The Portland isolate and one New Delhi isolate showed identical zymodemes, while others were

different from each other.

Meloni et al. (1988) studied 30 isolates; 25 from humans, 4 from cats and one from a rat. Twenty five isolates were obtained from Western Australia, and another 5 isolates were from Southern Australia, Queensland, Papua New Guinea, and the United States. Analyzing 10 enzymes including GPI, G6PD, MDH and ME, with starch gel electrophoresis, they could distinguish 13 different zymodemes among the 30 isolates.

Antigenic difference among G. duodenalis isolates has been demonstrated using various techniques. Korman et al. (1986) studied the agglutination of Giardia by anti-WB rabbit serum. Four human isolates were tested. The serum agglutinated only homologous WB trophozoites. The other 3 isolates, all of Jerusalem origin, did not react.

Unger and Nash (1987) studied cross-reactivity of anti-Giardia rabbit sera with 8 isolates. Antisera were raised against 11 isolates. Using immunofluorescence technique, homologous sera stained only the surface of trophozoites while heterologous sera stained whole cells with diffused internal fluorescence. They interpreted this result as different antigens were located on the surface while common antigens were located inside the cells.

Nash and Keister (1985) compared surface antigens of 19 G. duodenalis isolates by PAGE of surface-labeled trophozoites. Isolates were obtained from 15 human patients, one cat, one guinea pig, and two beavers. The electrophoretic migration patterns of labeled surface materials from the isolates differed in varying degrees from each other. At least 9 different patterns were observed among 19 isolates.

Meloni and Thompson (1987) showed a difference in nutritional requirements between human isolates and dog isolates by *in vitro* culture using one type of medium. De Jonckheere and Gordts (1987) showed differences in sensitivity against RNA virus among 38 *Giardia* isolates by virus transfection.

All of the above mentioned findings indicate that *Giardia* isolates referred to as *G. duodenalis* may include subspecies, and that it is important to examine the difference in the genetic or immunological properties of isolates when we study cross-transmission of *Giardia* between different animals. The definitions of *G. duodenalis*, suggested by Filice(1952), may not be perfect to define valid species of *Giardia*.

Several authors compared *G. duodenalis* isolates obtained in a single geographic area. Grant and Woo (1987b) compared 3 *G. duodenalis* isolates from small rodents in Southern Ontario, Canada. By cross-transmission experiments using 5 recipient animals, they showed that these three isolates were different species with distinct host specificities. Korman *et al.* (1986) compared 4 human isolates obtained in Jerusalem, Israel, by the isozyme analysis and by the agglutination response to rabbit anti-WB sera. Only two isolates were identical, and the other two isolates were different from each other. Baveja *et al.* (1986) analyzed 3 human isolates obtained in New Delhi, India, by starch gel electrophoresis of isozymes. Each of the 3 isolates showed different zymodemes. Meloni *et al.* (1988) compared 25 isolates obtained in Western Australia by isozyme electrophoresis using starch gel. Their samples include 21 human isolates, 3 cat isolates and one rat isolate, and they observed 13 different zymodemes among 25 analyzed isolates.

These studies reported by Korman et al. (1986), Baveja et al. (1986), and Meloni et al. (1988) showed that there is no obvious geographic correlations among isolates. Although some isolates from a single geographic area showed common characteristics, in most cases isolates from the same area had very different characteristics.

To study if the transmission of Giardia may occur among different animals in the natural environment, we compared biological characteristics of 7 Giardia isolates obtained from different animals in Southern Alberta, Canada. WB (ATCC 30957), an established human strain geographically originating in Afghanistan, was used as a standard. These isolates were compared in their general morphology, dimensions, DNA fragment patterns, zymodemes, surface antigens and intracellular pH.

2. Materials and Methods

2.1 Elimination of bacterial contamination

The antimicrobial susceptibility of contaminating bacteria was tested by the Kirby-Bauer method. A blood agar plate was inoculated with 0.1 mL of medium from contaminated WB culture. Whatmann 3MM filter paper cut into discs of 7 mm diameter was sterilized by autoclaving. To these discs, 20 μL of each of the following antibiotic solutions were applied; gentamicin, ticarcillin, penicillin G, and streptomycin. Three different concentrations, 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL, were used for each antibiotic. The discs were placed on the agar plate inoculated with WB culture medium. After incubation at 37°C overnight, the diameters of bacterial growth inhibition were measured. The antimicrobial susceptibility of bacteria was determined by the formation of clear zones around discs.

Based on the results of the antimicrobial susceptibility test, bacterial contamination in Giardia culture was eliminated as follows: Trophozoites freshly harvested from a 15 mL culture tube were transferred into a new sterile 15 mL tube and washed once in 50 mM phosphate-buffered saline pH 7.0 (PBS) and then centrifuged. The pellet was resuspended in 2 mL of PBS containing 2 mg penicillin G, 2mg gentamicin and 5 mg ticarcillin, and incubated at 37°C for at least 1 h.

This tube was filled with fresh medium and incubated again at 37°C. Four days later, bacterial contamination was tested by inoculating culture medium on trypticase soy agar supplemented with 0.5% yeast extract (TSA-Y) and in fresh culture medium without antibiotics.

The plates were incubated at 37°C for 2 weeks under both aerobic and anaerobic conditions. The broth cultures were incubated at 37°C in a CO_2 incubator.

2.2 Giardia isolates

The original hosts and locations of the Giardia isolates used in this study are listed in Table 1. WB (ATCC 30957) was obtained from the American Type Culture Collection. This is an established strain originally isolated from a giardiasis patient who contracted the infection in Afghanistan (Smith et al. 1982). Other isolates were established in culture as follows by Wallis and Wallis (1986). Original cysts were extracted from faeces of host animals; H7 and H8 were from giardiasis patients in Calgary, and other 5 isolates were from mammalian hosts found in various locations in Southern Alberta (Fig. 2). These cysts were administered to recipient animals to obtain trophozoites. As recipients, a Giardia-free wild deer mouse (Peromyscus manicultatus) was used for PB1 isolate, and Giardia-free mongolian gerbils (Meriones unguiculatus) were used for other isolates. The wild deer mouse was livetrapped and cleared of intestinal protozoa with metronidazole before infection. Trophozoites were isolated from the duodenums of infected animals and established in culture using TYI-S-33 medium.

Isolates were routinely cultured at 37°C in 15 mL glass tubes filled with modified TYI-S-33 medium (Keister 1983) using casein digest peptone instead of trypticase peptone (Table 2). After sterilization by autoclaving or filtration, all components were mixed. Penicillin G sodium was obtained from Ayerst, Montreal, Canada, and ticarcillin clavulanic acid disodium was from Beecham Labs, Tenn., U.S.A.

Table 1 Sources of *G. duodenalis* isolates

STRAIN	ORIGINAL HOST	DATE	LOCATION
WB	Human	-	Afghanistan
H7	Human	85-08-03	Calgary
H8	Human	-	Calgary
B5	Beaver	85-06-19	Sibbald Meadows Pond Kananaskis Country, 65 km West of Calgary
PB1	Beaver	84-11-23	Lusk Cr. Pond Kananaskis Country, 80 km West of Calgary
MR4	Muskrat	85-09-20	Same as B5
S1	Sheep (domestic)	85-07-02	Farm near Strathmore 45 km East of Calgary
D3	Dog	84-08-16	Calgary Animal Shelter

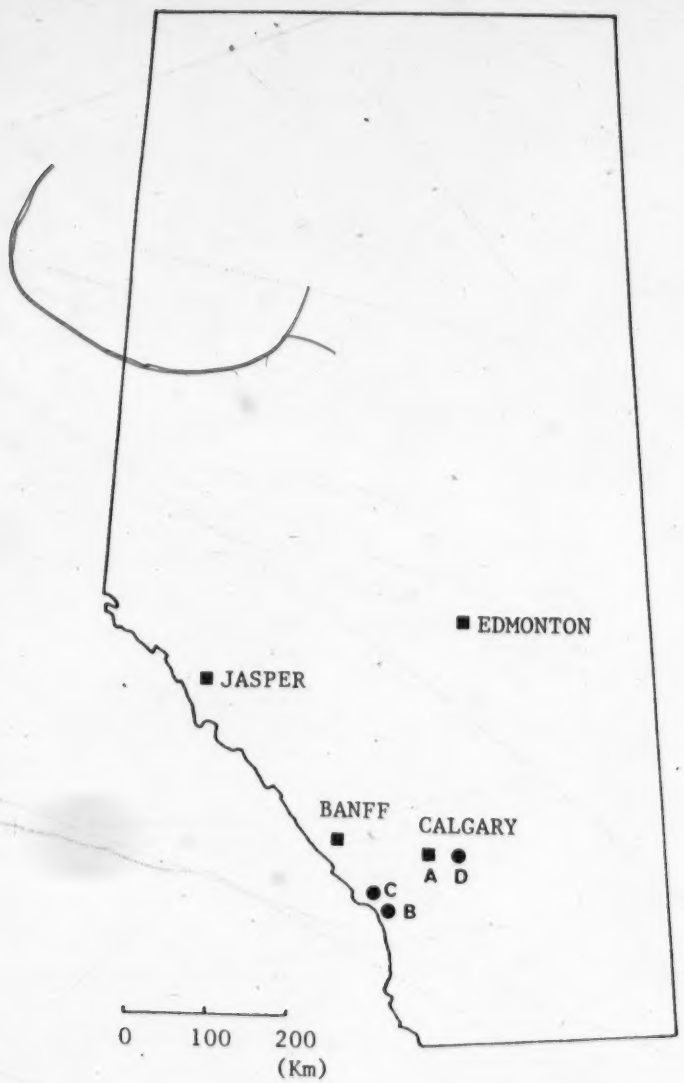


Figure 2 Collecting locations of G. duodenalis isolates in Southern Alberta.

H7, H8 and D3 were collected at point A.

B5 and MR4 were colleced at point B.

PB1 and S1 were collected at point C and D, respectively.

Table 2 Preparation of modified TYI-S-33 medium

Table 2 has been removed because of the unavailability of copyright permission.

Subculture was prepared every 3 or 4 days. Medium was discarded from the original culture tube and 3 mL of sterile PBS was added. This tube was chilled in ice for 10 min and shaken to dislodge the trophozoites attached to the glass surface. After shaking, 0.1 mL of the cell suspension was transferred into a new tube filled with fresh medium and incubated at 37°C as subculture.

To harvest trophozoites for analysis, cell suspensions prepared as above were centrifuged at 1,000 x g for 5 min. The pellets were then washed twice with PBS by centrifuging, and resuspended in an adequate volume of PBS or other buffers.

Trophozoites were cryopreserved by resuspending cells in 2 mL of fresh medium with 10% dimethyl sulfoxide (DMSO) without antibiotics. The trophozoites were freshly harvested from confluent growth culture in a 15-mL glass tube. One mL aliquot was transferred into a 3 mL plastic tube and cooled gradually from room temperature to -70°C taking at least 3 h, and finally stored in liquid nitrogen. Since a sudden change of temperature may cause cell death, the trophozoites were cooled gradually.

2.3 Chemicals

Reagents were obtained from Sigma Chemical Co. (St. Louis, Missouri) unless otherwise indicated.

2.4 Protein determination

The concentration of protein was determined by the modified Lowry method. Thirty μL of the sample were mixed with 1.0 mL of $CuSO_4$ reagent and left to stand for 10 min. Then 100 μL of 1.0 N Folin

solution was added in the mixture and shaken immediately. After 30 min, protein concentration was determined by reading the absorbencies at 750 nm. A standard curve was prepared using bovine serum albumin (BSA) solution (1 mg/mL) in the range from 5 to 30 μg protein. The $CuSO_4$ reagent was prepared by mixing 100 μL of 350 mM sodium potassium tartrate, 100 μL of 200 mM $CuSO_4$, and 10 mL of 2.0% sodium carbonate in 100 mM NaOH.

2.5 Cell counts

The suspension of Giardia trophozoites or other cells used in this study was mixed with an equal volume of Türk solution and placed in a haemocytometer. The number of cells was counted using a microscope and the cell number per mL was calculated. Türk solution was prepared by mixing 1.0 mL of 1.0% aqueous gentian violet, 2.0 mL of glacial acetic acid and 97.0 mL of distilled water. This mixture was filtered before use.

2.6 Scanning electron microscopy

Each Giardia isolate was grown in a 50 mL propylene tube containing 50 mL of medium and a piece of microscope slide cover glass (22 X 40 mm) by inoculating 0.1 mL of cell suspension as described in section 2.2. The cells harvested from confluent growth were used for inoculation to minimize the morphological differences both within an isolate and among isolates which may be caused by the different ages of the cells. Each culture was incubated at 37°C for 2 days to obtain the monolayer of trophozoites on the cover glass.

To prepare specimens for scanning electron microscopy (SEM), the cover glass was transferred to a petri dish and flooded with 2.5% glutaraldehyde in 0.1 M cacodylate-HCl buffer (pH 7.3) containing 5 mM $CaCl_2$, and left for 30 min at room temperature to fix trophozoites. The cover glass was then rinsed 3 times with 0.1 M cacodylate buffer and flooded with 0.1% osmium tetroxide in the same buffer to postfix trophozoites for 30 min at room temperature.

The cover glass was rinsed again 3 times with cacodylate buffer and processed for stepwise dehydration in ethanol. The dehydration sequence was as follows; 25% ethanol 15 min, 50% ethanol 15 min, 75% ethanol 15 min, 90% ethanol 15 min 2 changes, and 100% ethanol 15 min 2 changes. Finally, the trophozoites on the cover glass were dried by the critical point drying method (Anderson 1951) and a small piece of the cover glass was cut and mounted on a stub. After spulter-coating the trophozoites with gold, photographs were taken using a Philips SEM 505 scanning electron microscope with 1.05×10^3 magnification.

Dimensions of trophozoites were analyzed according to the method of Grant and Woo (1978b). The length and width of the trophozoites were determined by measuring the first 40 intact cells encountered in the SEM photographs of each isolate. The unit bar (10 μm) automatically printed in the photograph was used as the unit length for measurement. The variability of this unit bar was within $\pm$ 10% depending on the experimental conditions such as the instrument used or the duration of measurement. The maximum width at the level of nuclei was defined as width. The maximum length from the anterior end to the posterior end of trophozoite body was defined as length.

2.7 DNA analysis

2.7.1 Isolation of DNA

DNA was isolated from a one-litre culture of each Giardia isolate. The culture was tested for bacterial contamination by inoculating 0.1 mL of medium on TSA-Y and incubating at 37°C for 2 weeks under both aerobic and anaerobic conditions.

Trophozoites of each isolate were harvested from 4 of 250 mL glass culture bottles. The bottles were chilled in ice for 10 min and shaken to dislodge trophozoites attached to the glass surface. The medium was centrifuged at 1,000 x g for 10 min to collect trophozoites, and after washing twice in cold PBS by centrifuging, the harvested cells were suspended in 2 to 4 mL of 50 mM Tris-HCl buffer (pH 7.9) containing 50 mM NaCl and 10 mM EDTA to store at -20°C until the next procedure.

To isolate DNA, 12 mL of lysis solution was added to the thawed cell suspension and the mixture was incubated overnight at 37°C. This lysis solution consists of 0.1 M Tris-HCl (pH 7.9), 0.1 M NaCl, 0.05 M EDTA, 0.5% sodium dodecyl sulfate (SDS), and 200μg/mL proteinase K. Then the lysate was mixed with one-half volume of redistilled phenol saturated with 10 mM Tris-HCl (pH 7.9) and one-half volume of chloroform-isoamyl alcohol (24:1). The mixture was rotated gently for 30 min at room temperature. To separate phases, this mixture was centrifuged for 10 min at 1,000 x g and the aqueous phase was transferred into a new tube. An equal amount of chloroform-isoamyl alcohol (24:1) was added to the aqueous phase, and the mixture was rotated gently for 15 min at room temperature.

After centrifuging, the aqueous phase was transferred into a new tube and incubated with RNase (100 μg/mL) overnight at 37°C. This RNase was heated at 80°C for 30 min before use to inactivate contaminating DNase. Proteinase K (200 μg/mL) was then added in the aqueous phase to incubate for 2 h at 37°C. The mixture was extracted with an equal volume of chloroform-isoamyl alcohol (24:1), and DNA in the aqueous phase was ethanol precipitated at -20°C by adding 0.1 volume 3.0 M sodium acetate (pH 5.0) and 2.5 volumes cold 95-100% ethanol. The precipitate was dissolved in 1.0 mL of 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl and 1 mM EDTA, and dialyzed overnight against the same buffer. DNA was ethanol precipitated again and resuspended in 0.5 mL to 1.0 mL of the same buffer for further analysis.

2.7.2 Endonuclease digestion and hybridization

The following three restriction endonucleases were used to digest DNA: BamHI (New England Biolabs), HindIII (Boehringer Mannheim), and PstI (Boehringer Mannheim). Approximately 1 μg of DNA per sample was digested with 2 to 5 units of enzyme (Maniatis et al. 1982a) under conditions suggested by the manufacturer, and electrophoresed overnight at 30V (1.4 V/cm) in a 1.0% agarose gel (Maniatis et al. 1982b). HindIII-digested λ phage DNA (Bio-Rad Labs) was used as molecular size standards. After ethidium bromide staining of the gel, DNA was transferred to nitocellulose filters (Sartorius) according to the method of Southern (1975).

^{32}P-labeled whole DNA probes were prepared by nick translation from DNA of 3 Giardia isolates, WB, H7 or D3, according to the method of Maniatis et al. (1982c). The activity of each probe was: 1.14×10^{7}

CPM/mL for WB probe, 1.76×10^7 CPM/mL for H7 probe, and 4.69×10^7 CPM/mL for D3 probe.

The DNA restricted with each of the above-mentioned endonucleases was hybridized with WB, H7 or D3 DNA probe under stringent conditions in 50% formamide at 37°C overnight using $10^6 - 10^7$ CPM of probe. The blot was then washed in 2xSSC with 0.2% SDS for 5 min at room temperature, in 2xSSC with 0.2% SDS for 30 min at 65°C twice, and in 0.2xSSC for 30 min at 65°C twice, successively. SSC consists of 8.765g/L of NaCl and 4.41 g/L of sodium citrate with the pH adjusted to 7.0 with acetic acid.

This blot was exposed to Kodak X-Omat AR film with two intensifying screens at -70°C overnight to autoradiograph. Although an approximately identical amount of DNA was electrophoresed in each lane, there was difference in the intensity of DNA bands appeared on the film. Therefore, to compare DNA fragment patterns of 8 Giardia isolates, each blot was exposed to films with various exposure times so that DNA from each isolate would reveal the bands of same intensity.

2.8 Isozyme analysis

2.8.1 Electrophoresis of isozymes

Electrophoretic migration patterns of glucose-6-phosphate dehydrogenase (G6PD, E.C. 1.1.1.49), malic enzyme (ME, E.C. 1.1.1.40), malate dehydrogenase NAD^+ (MDH, E.C. 1.1.1.37) and glucose phosphate isomerase (GPI, E.C. 5.3.1.9) were analyzed.

Trophozoites harvested from culture bottles were stored at -20°C as a pellet after centrifuging. To prepare cell lysate, the frozen cell pellet was thawed at 37°C and an equal weight of distilled water was

added. By the repetition of freezing at -70°C and thawing at 37°C 5 times, the trophozoites were lysed. After centrifuging at 13,000 x g for 5 min in a microcentrifuge, 20 μL aliquots of the supernatant were stored at -70°C as cell lysate samples.

For electrophoresis, 2 to 4 μL of lysate was applied in each lane of 12.5% or 9.0% polyacrylamide gel and electrophoresed at 3 mA constant current overnight at 4°C. Gels and reservoir buffers were prepared according to the procedures of Laemmli (1970) except that SDS was omitted.

After electrophoresis, locations of ME, MDH and GPI were visualized by overlaying developer reagents (Table 3) over the gels and incubating at 37°C in the dark until color was developed.

To detect G6PD, electrophoresed protein was transferred to a nitrocellulose filter by the method of Towbin et al. (1979), except that methanol was omitted from the transfer buffer. Constant current of 195 mA was applied at 4°C for 1 h. The enzyme was visualized by pouring the developer reagent (Table 3) over the nitrocellulose filter and incubating at 37°C in the dark for 20 min.

2.8.2 Determination of Michaelis constants

To determine the apparent Michaelis constants (Km) of G6PD, 100 μL of trophozoite lysate, prepared as described in section 2.8.1 and diluted 100 times with 100 mM Tris-HCl (pH 7.5), was added to the enzyme substrate mixture. This substrate mixture was prepared by mixing 100 μL of 300 mM $MgCl_2$ $6H_2O$, 0.2 mM NADP monosodium salt, 0.6 mL of 1% Triton X-100 in 100 mM Tris-HCl (pH 7.5), and 100 μL of substrate solution. As substrate solutions, a series of 5 doubling dilutions of

Table 3 Components of developer reagents

Table 3 has been removed because of the unavailability of copyright permission.

glucose-6-phosphate starting from 2 mM was used.

After the addition of lysate, the reaction mixture was incubated at 37°C for 30 min with gentle shaking. The fluorescence produced by a reduced form of NADP was measured at 340 nm with a spectrophotometer. A reaction mixture in which lysate was replaced with 100 mM Tris-HCl (pH 7.5) was used as a blank control. Triplicate tests were performed for each substrate dilution and the Km was obtained from Lineweaver-Burk plot.

To determine the apparent Km of ME, substrate mixture was prepared by mixing 100 μL of 6.6 mM $MnCl_2$, 100 μL of 3.3 mM of NADP monosodium salt, 0.6 mL of 1% Triton-100 in 100 mM Tris-HCl (pH 7.5), and 100 μL of substrate solution. A series of 5 doubling dilutions of malic acid starting from 0.833 mM, neutralized with NaOH, was used as substrate solutions. The reaction mixture was processed as above.

2.9 Intracellular pH determination

Intracellular pH was measured according to the method of Grinstein et al. (1984a). Freshly harvested trophozoites were resuspended in 2 mL of choline$^+$ solution (pH7.2) to make 10^8 cells/mL of cell suspension. The choline$^+$ solution consists of 140 mM choline chloride, 10 mM glucose, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES.

The cells were then loaded with bis (carboxy-ethyl) carboxy fluorescein acetoxymethyl ester (BCECF) by incubation with 3 μL of BCECF (1 μmole/μL DMSO) at 37°C for 45 min. The ester form of fluorescein, which shows little fluorescence, diffuses across the cell membrane during incubation. Inside cells, this ester is hydrolyzed by

cytoplasmic esterases and releases fluorescein with strong fluorescence, which can serve as an indicator of intracellular pH (Pink et al. 1982).

After incubation with BCECF, 1.0 mL of cell suspension was washed once in fresh choline$^+$ solution by centrifuging and resuspended in 1.0 mL of the same solution. The fluorescence of this cell suspension was measured at 37°C in a Perkin-Elmer LS-5 fluorescence spectrophotometer attached to a Perkin-Elmer R-100 recorder. Excitation and emission wavelength were 500 and 530 nm, respectively. The pH corresponding to the intensity of this fluorescence was defined as the intracellular pH and determined from a calibration curve between pH and fluorescence. The calibration was carried out for each sample by disrupting the cells with 100 μL of 1.0% Triton X-100, followed by repetitive titration of the medium with 1.0 N HCl. Every time 3 μL of 1.0 N HCl was added, fluorescence and the pH of medium were measured. Intracellular pH was measured at least 3 times for each isolate and the average was calculated.

The fluorescence emitted by intracellularly-trapped fluorescein derivatives is usually decreased compared with the free dye because the spectra of the intracellular dyes are slightly red-shifted (Thomas et al. 1979). The correction factor for this red shift was determined by the method of Grinstein et al. (1984b). Cells were loaded with BCECF as described above and resuspended in 1.0 mL of K^+ solution with 5 different pH values ranging from 6.1 to 7.3. The formula of the K^+ solution is identical with that of the choline$^+$ solution except that choline chloride was isoosmotically replaced by KCl.

The cell suspension was placed in a cuvette in the spectrophotometer and treated with 0.7 μg/mL nigercin. The addition of nigercin causes rapid equilibration of the external and internal pH (Thomas et al. 1979). This is because the ionophore set Hi^+/Ho^+ is equal to Ki^+/Ko^+, so that if the cells are suspended in K^+ solution which contains approximately the same K^+ concentration as the cytoplasm, Hi^+ will follow Ho^+ (Grinstein et al. 1984b). Hi^+/Ho^+ or Ki^+/Ko^+ indicates the racio of H^+ or K^+ concentration inside cells and outside cells.

The fluorescence was recorded once the signal stabilized. Then cells were disrupted by the addition of 100 μL 1% Triton X-100 to record the fluorescence of released BCECF. The ratio between the original fluorescence and the fluorescence after the addition of nigercin was calculated to find the effect of a red shift on the intracellular pH determination.

2.10 Preparation of anti-WB monoclonal antibody

2.10.1 Cell fusion

WB trophozoites, freshly harvested under sterile conditions and washed twice in sterile PBS, were resuspended in sterile PBS to make 10^8 cells/mL of suspension. Then 0.2 mL of this suspension was mixed thoroughly with an equal volume of complete Freund's adjuvant using a sterile polypropylene syringe, and injected into a 6-week-old female BALB/c mouse intraperitoneally. The remainder of the cell suspension was prepared in 0.5 mL of aliquots and stored at -20°C for further immunization.

Three weeks later the mouse was inoculated again with 2×10^7 cells in PBS intraperitoneally, followed by inoculation of the same dose of cells in the same manner every 2 weeks 3 times. The mouse was then rested for 3 weeks and boosted intraperitoneally with 5×10^7 cells in PBS. After 3 days, 0.1 mL of blood was taken from the tail of the mouse to analyze for antibody titer by a double antibody enzyme-linked immunosorbent assay (ELISA). When the antibody titer was greater than 1 in 1,000, the mouse was sacrificed for cell fusion.

The culture of SP 2/0 myeloma cells was restored from cryopreserved cells 5 days before cell fusion. RPMI 1640 (Flow Laboratories Ltd) supplemented with 50 μM 2-mercaptoethanol, 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, and 200 μg/mL gentamicin was used as medium. The cells were grown at 37°C and maintained in log-phase (10^5 cells/mL).

On the day of cell fusion, myeloma cells were harvested and washed once in RPMI 1640 containing 200 μg/mL gentamicin (RPMI+GE) by centrifuging at 1,000 rpm for 10 min at room temperature. The cells were resuspended in 20 mL RPMI+GE. Spleen cells from the immunized mouse were washed once in the same way as myeloma cells and resuspended in 5 mL RPMI+GE. The number of cells was counted as described in section 2.5 using Turk solution, and myeloma and spleen cells were mixed at a ratio of 1:5 in a 50 mL sterile propylene tube.

This cell mixture was centrifuged at 1,000 rpm for 10 min at room temperature, and the medium was removed completely by aspiration. To the cell pellet, 0.5 mL of prewarmed (37°C) polyethylene glycol solution (PEG) was added over a 1 min period while stirring gently with a pipette tip. PEG was prepared by mixing 2g polyethylene glycol [MW

4,000 Merck #ART9727], 2 mL RPMI 1640, and 0.2 mL DMSO in a glass tube and autoclaving the mixture for 15 min.

Then 10 mL of prewarmed (37°C) RPMI+GE was added slowly over a 5 min period to this tube with gentle mixing. After incubation at 37°C for 30 min, another 10 mL of warm RPMI+GE was added into this tube. The cells were centrifuged at 1,000 rpm for 10 min and washed once in the same medium.

The final pellet was resuspended in hybridoma medium to make the final concentration of 10^5 myeloma cells/mL, and plated 1.0 mL/well in five of 24-well tissue culture plates. The hybridoma medium was prepared by supplementing RPMI 1640 with 50 μM 2-mercaptoethanol, 0.45 g/mL glucose, 20% FCS, 10% NCTC-109 (Gibco #320-1340), 30 μg/mL oxalacetate, 10 μg/mL sodium pyruvate, 40 mU/mL bovine insulin, 100 μM hypoxanthine, 30 μM thymidine, 0.5 μM aminopterin, 2 mL L-glutamine, 100 μg/mL penicillin G, and 100 μg/mL streptomycin.

Red blood cells from an unimmunized BALB/c mouse were suspended in hybridoma medium to 10^5 cells/mL, and 1.0 mL of this cell suspension was added in each well containing the fused cells to be used as feeder cells. The plates were incubated at 37°C for 17 days until visible colonies were formed. More than 100 visible colonies in total were obtained. The medium from wells containing colonies was tested by ELISA to detect antibody against Giardia. When medium showed antibody production, hybridoma colonies were picked up from wells and transferred into new 24-well plates filled with 2 mL/well of HT medium. To prepare HT medium, RPMI 1640 was supplemented with 100 μM hypoxanthine, 30 μM thymidine, 50 μg/mL 2-mercaptoethanol, 10% FCS, 2 mM L-glutamine, 100 μg/mL penicillin G and 100 μg/mL streptomycin.

These culture plates were incubated for 3 weeks and the medium was tested again by ELISA. Although most of the hybridoma cell lines showed positive reactions, 19 colonies which showed the strongest reaction with ELISA (Optical absorbencies at 405 nm greater than 1.9) were chosen for further cloning. Cells were cloned by limiting dilution using HT medium, and red blood cells were used as feeder cells as described above. When visible colonies were formed, the hybridoma cells were cloned again.

After the second cloning, the colony of each cloned cell line was transferred to a 50 mL-tissue culture bottle containing 5 mL of HT medium, and incubated at 37°C. Fresh medium was added gradually in this culture bottle over a period of 2 weeks to maintain the cell density at 10^5 cells/mL. When the total cell number reached 10^7, the cells were harvested by centrifuging at 1,000 rpm for 10 min and resuspended in 2 mL of RPMI 1640 containing 20% FCS and 10% DMSO. Two aliquots, each containing one mL of cell suspension, were made and cooled gradually to -70°C preventing cell death as described in section 2.2 for cryopreservation. The frozen hybridoma cells were stored in liquid nitrogen until use.

2.10.2 Enzyme-linked immunosorbent assay

Soluble Giardia antigens were prepared by sonicating WB trophozoites in PBS for 30 sec at least 4 times in ice, followed by centrifuging at 13,000 x g for 5 min to remove insoluble material. The protein concentration of the supernatant was determined as described in section 2.4, and the supernatant was diluted with PBS to 40 μg protein/mL.

A 96-well tissue culture plate was coated with WB antigen by applying 100 μL of soluble antigen to each well. The plate was sealed and incubated overnight at 4°C. The antigen solution was shaken out, and the plate was rinsed twice with 100 mM phosphate buffered saline pH 7.0 containing 0.1% Tween-20 (PBS-T).

To test antibody production, 100 μL of the medium from the hybridoma culture were added to a well. EIA diluent (PBS containing 0.5% BSA and 1% Tween-20) was used as a control. The plate was incubated at 37°C for 30 min. The plate was rinsed three times with 100 μL of PBS-T, and 100 μL of the second antibody solution was added to each well. Alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Bio-Rad Lab Ltd) diluted 100 times with EIA diluent was used as the second antibody solution.

After incubation at 37°C for 30 min, the plate was rinsed 3 times with PBS-T, and 100 μL of alkaline phosphatase color development reagent (Bio-Rad Lab Ltd.) were added in each well. This plate was incubated at room temperature for 30 to 60 min and the absorbencies was measured at 405 nm with an EIA microtitration plate reader.

2.10.3 Ascites fluid production

A 6-week-old female BALB/c mouse was primed by the intraperitoneal injection of 0.5 mL Pristane (2,6,10,14-tetramethyl penadecane, Aldrich), 5 days before the injection of hybridoma cells.

A hybridoma cell line 42A3 which produces monoclonal antibody against WB was restored in culture from cryopreserved cells using 30 mL of HT medium. This culture was expanded to 200 mL by adding fresh

medium gradually while maintaining the cell density at 10^5 cells/mL. After 6 days, cells were harvested and 10^7 cells, resuspended in 0.3 mL of RPMI 1640 were injected peritoneally into the primed mouse.

The mouse was sacrificed 11 days after the injection of hybridoma cells as its abdomen was swollen. Ascites fluid was drawn from the peritoneal cavity using a 21G needle and 10 mL syringe. The fluid was transferred into a 7 mL glass tube and left to stand for 1 h at room temperature to allow fibrin clot. The fibrin was removed by centrifuging at 1,000 x g for 10 min. The supernatant was then transferred into a new tube and incubated at 50°C for 30 min to inactivate complement and protease. The heat-treated ascites fluid was stored at -70°C until use.

2.11 Agglutination assay

Freshly harvested Giardia cells of each isolate were resuspended in PBS to 9 x 10^5 cells/mL, and 50 µL of each suspension were added to wells of a 96-well tissue culture plate. One µL of mouse ascites fluid containing anti-WB monoclonal antibody (monoclonal ascites fluid) was added to each well, and the plate was shaken gently for 30 min at room temperature. As a control, 1 µL of ascites fluid accumulated by injecting SP2/0 myeloma cells (Ascites control SP2/0 b27, Appel, Cochranville, PA) was added to a well containing WB trophozoites. Agglutinated trophozoites were photographed using a phase contrast microscope equipped with a photographic apparatus.

2.12 Indirect immunofluorescence microscopy

A microscope slide was coated with gelatin by dipping in 0.5% hot gelatin and drying overnight at room temperature. Trophozoites of 8 Giardia isolates, fixed in 0.37% formalin for 30 min and washed once in PBS, were applied on the gelatin-coated microscope slide as small dots using a one-μL micropipet and dried for 30 min at room temperature.

The cells on the slide were fixed in acetone for 10 min at room temperature and rinsed with PBS. The monoclonal ascites fluid was diluted 1:100 in PBS, and applied on the fixed cells with a microbiological loop. This slide was incubated at 37°C for 30 min in a petri dish containing wet tissue to prevent drying. Ascites control, diluted in the same way as above, was used as control.

After the incubation, the ascites fluid on the slide was washed off with PBS and the slide was rinsed in PBS for 5 min twice with stirring, followed by drying at room temperature for 30 min.

Fluorescein isothiocyanate conjugated anti-mouse goat IgG, diluted 1:35 in PBS, was applied on cells with a microbiological loop. This slide was incubated at 37°C for 30 min in a humid atmosphere as described above. The slide was rinsed twice in PBS for 5 min with stirring, and dried for 30 min at room temperature. The dried slide was mounted using buffered glycerol solution, and observed under a fluorescence microscope. The buffered glycerol solution was prepared by mixing 1.0 mL of 0.5M carbonate buffer (pH 9.5) and 9 mL of glycerine. Kodak Tri-X pan 400 was used for photography.

2.13 Immunoprecipitation assay

2.13.1 Radioiodination of WB trophozoites

Trophozoites of WB isolate, freshly harvested from a 15 mL-glass tube, were washed once in PBS and resuspended in 75 μL of 0.2 M phosphate buffer (pH 7.0). In this suspension, 2 μL of $Na^{125}I$ (4GBg/mL), 20 μL of 0.1 mM KI, 20 μL of lactoperoxidase (1 unit/20 μL of 0.2 M phosphate buffer pH 7.4) and 20 μL of diluted H_2O_2 were added, and the mixture was left to stand at room temperature. The diluted H_2O_2 was prepared by mixing 10 μL of concentrated H_2O_2 with 10 mL of H_2O just before iodination.

Seven min later, 20 μL of lactoperoxidase and 20 μL of diluted H_2O_2, prepared as above, were added and the mixture was left to stand for 10 min. Then 20 μL of 25% NaN_3 and 25 μL of KI solution (0.1 mg/mL) were added to stop the reaction, and the cells were washed once in PBS by centrifuging.

2.13.2 Electrophoresis of immunoprecipitated antigens

The pellet of ^{125}I-labeled cells was resuspended in 0.5 mL RIPA buffer and sonicated for 30 sec 3 times in ice. RIPA buffer was prepared by supplementing 10 mM Tris-HCl buffer (pH 7.4) with 0.14 M NaCl, 1.0% sodium dodecyl cholate, 1.0% Triton X-100, 0.6% SDS, 1 mM phenyl methyl sulfonyl fluoride, and 10,000 units trasylol (Miles Pharmaceutics).

Sonicated cells were left to stand for 20 min at room temperature, and centrifuged at 13,000 x g for 5 min in a microcentrifuge to remove insoluble debris. The supernatant was transferred into a new tube, and rotated for 1 h at 4°C with 10 μL of monoclonal ascites fluid.

As a control, 10 μL of the ascites control were used.

Fifty μL of protein A-Sepharose suspension (PA-Sepharose) was added in the mixture of sonicated cells, and the tube was rotated again for another 10 min at 4°C. To prepare PA-Sepharose, 20 μL of dry protein A-Sepharose CL-3B (Pharmacia) was suspended in 0.5 mL of RIPA buffer and rotated for 2 h at 4°C to make homogeneous slurry.

After centrifuging at 13,000 x g for 2 min, immunoprecipitated antigens were washed in 1.0 mL of RIPA buffer 5 times. The precipitate was washed again in 1.0 mL of distilled water, and mixed with an equal volume of final sample buffer (FSB) for electrophoresis. FSB was prepared by mixing 0.757 g Tris base, 2.0 g SDS, 10.0 mL glycerol, 1 mg bromophenol blue, and 5.0 g 2-β-mercaptoethanol in 100 mL of deionized distilled water. The sample was boiled for 3 min and cooled in ice, and centrifuged to remove Sepharose. The supernatant was electrophoresed by 7.0% or 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at a constant current of 40 mA for 2 h according to the method of Laemmli (1970) at room temperature with water cooling.

As molecular markers, the low molecular weight calibration kit (Pharmacia) and the high molecular weight markers (SDS-PAGE Standards high molecular weight, Bio-rad) were used. The low molecular weight markers were phosphorylase b (94,000), bovine serum albumin (BSA; 67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400). The high molecular weight markers were myosin (200,000), β-galactosidase (116,250), phosphorylase b (95,500), BSA (66,200), ovalbumin (45,000). Molecular weights are shown in parentheses. The molecular weights of phosphorylase b and BSA in the high molecular weight markers are

different from those in the low molecular markers probably because the manufacturers of these markers used different sources to purify the proteins or they used different methods to determine the molecular weights.

After electrophoresis, the gel was stained with Coomassie blue to visualize proteins, and destained with a mixture of 5.0% methanol and 7.0% acetic acid. Coomassie blue R-250 1.25 g was dissolved in 225 mL methanol and 45 mL acetic acid, and made up to 500 mL with H_2O to make the staining solution.

The gel was dried on 3MM Whatmann filter paper under vacuum with heating. The dried gel was exposed to Kodak X-Omat AR film with one intensifying screen at -70°C for several hours to overnight to autoradiograph. The molecular weights of antigens were calibrated from molecular standards.

2.14 Western blot assay

Soluble Giardia WB antigen, prepared as described in section 2.10.2, was mixed with an equal volume of FSB and boiled for 3 min, then cooled in ice. The sample was electrophoresed at 40 mA for 2 h by 12.5% SDS-PAGE as described in section 2.13.2. Soluble antigen equivalent to 100 μg of protein was applied to each lane. The low molecular weight markers described in section 2.13.2 were used as standards.

After electrophoresis, proteins in the gel were transferred to a nitrocellulose filter by the method of Towbin et al. (1979). Constant current of 195 mA was applied for 1 h in transfer buffer containing 20 mM Tris base, 150 mM glycine and 20% methanol. The nitrocellulose filter was stained in Amido Schwartz and destained in H_2O to visualize

proteins, then stored at -20°C until use.

Before reacting with monoclonal ascites fluid, the blot was incubated in 2.0% ovalbumin in TSA (OA-TSA) for 10 min at room temperature to block protein binding sites. TSA was prepared by dissolving 6.055 g tris base, 0.01 g NaCl, and 3.33 mL HCl in H_2O to make 1 L. Then the blot was incubated with monoclonal ascites fluid, diluted 1:100 in OA-TSA, at room temperature overnight. Ascites control described in section 2.11 was used as a control.

After incubation, the blot was washed in TSA for 1 h with several changes, and reacted with 5×10^6 CPM of ^{125}I-labeled protein A (Pharmacia) in 200 mL of OA-TSA at room temperature with gentle shaking. The reacted blot was washed in TSA for 2 h with several changes, and rinsed once in water. The air-dried blot was exposed to Kodak X-Omat AR film with 2 intensifying screens at - 70°C to autoradiograph. The molecular weights of antigens were calibrated from molecular standards.

A blot of undenatured antigens was prepared by electrophoresing WB soluble antigens in 12.5% PAGE without SDS. Soluble antigens were mixed with an equal volume of FSB without SDS, and 100 μg of protein antigen were applied per lane. This blot was reacted with the ascites fluid containing anti-WB monoclonal antibody and the control ascites fluid in the same way as described above.

3. Results

3.1 Elimination of bacterial contamination

The original cultures of G. duodenalis isolates used in this study were contaminated with bacteria. To eliminate these bacteria, the antimicrobial susceptibility of the contaminating bacteria was tested by the Kirby-Bauer method. The bacteria were susceptible to gentamicin, ticarcillin and penicillin, although they could grow in Giardia culture medium supplemented with these antibiotics (Table 2). Ticarcillin was the most effective antibiotic in eliminating bacterial contamination. The bacteria were resistant to streptomycin.

Since the contaminating bacteria were still susceptible to the three antibiotics routinely used in culture medium, the amount of these antibiotics was increased three times more than the regular concentration. Namely, the medium was supplemented with penicillin 600 μg/mL, gentamicin 600 μg/mL, and ticarcillin 1.50 mg/mL, but this method was ineffective to eliminate bacterial contamination. The bacteria grew in the cultures after 4 days probably because the antibiotics were unstable at 37°C in culture medium. Even adding antibiotics (penicillin 200 μg/mL, gentamicin 200 μg/mL, and ticarcillin 500 μg/mL) to the culture tubes every 6 h did not eliminate the bacteria.

Finally, trophozoites, freshly harvested from culture, were treated with antibiotics in 50 mM phosphate-buffered saline pH 7.0 (PBS) at 37°C for at least 1 h. The concentrations of the antibiotics used were penicillin 1.0 mg/mL, gentamicin 1.0 mg/mL, and ticarcillin 2.5 mg/mL, respectively. This treatment eliminated contaminating

bacteria, and axenic cultures of 8 Giardia isolates were obtained. Subcultures prepared from these trophozoites did not show any visible bacterial growth after 4 days' incubation.

The complete elimination of bacteria was confirmed by subsequent inoculation of medium from these cultures on trypticase soy agar supplemented with 0.5% yeast extract and in fresh medium without antibiotics. The plates did not show any visible bacterial growth after 2 weeks' incubation at 37°C under both aerobic and anaerobic conditions. Likewise, the broth cultures did not show visible bacterial growth although the medium was not changed for 2 weeks.

3.2 Morphological assay

3.2.1 General morphology

Trophozoites of 7 isolates collected in southern Alberta and WB were observed by scanning electron microscopy (SEM) to see if they were of the G. duodenalis type, and if there was any distinct morphological difference among them.

The general morphology of 8 isolates was indistinguishable. Trophozoites of each isolate had a teardrop-shaped body surrounded by a ventrolateral flange (VLF) and flagella emerging from anterior, ventral, and caudal regions (Fig. 3).

The dorsal surface of most trophozoites was smooth (Fig. 3), but some had a rough surface (Fig. 4A). A pit-like depression (Fig 3-D3), sometimes elongated to form a slit (Fig. 4B), was observed in the centre or along the midline of bodies of many trophozoites. This depression or slit was commonly observed in all isolates.

Figure 3 Low-magnification SEM photographs showing general morphology of trophozoites of G. duodenalis isolates. Each Giardia isolate was grown on a piece of microscope cover glass to obtain a monolayer of trophozoites. The trophozoites on the cover glass were fixed with 2.5% glutaraldehyde for 30 min, and postfixed with 0.1% osmium tetroxide for 30 min. The fixed trophozoites were dehydrated in ethanol and dried by a critical point drying method. After coating the trophozoites with gold, photographs were taken using a Philips SEM 505 scanning electron microscope at a magnification of 1.05×10^3. Bar represents 10 μm. (———)

WB

H7

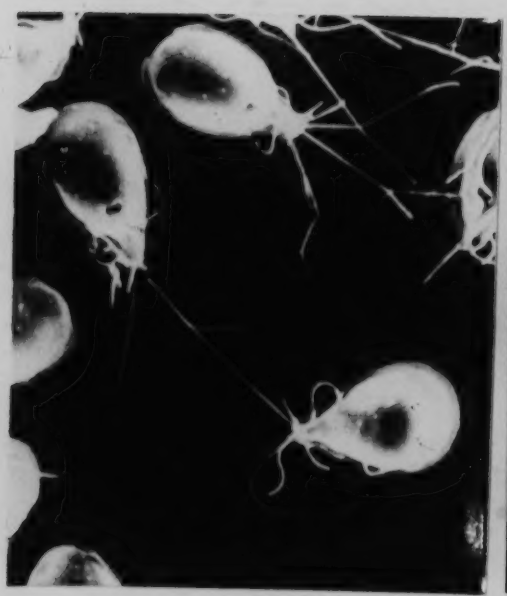
H8

B5

PB1

MR4

S1



D3

Figure 4 SEM photographs showing a trophozoite with a rough surface and a trophozoite with slit on surface.
Trophozoite A has a rough dorsal surface with knoblike protrusions. Trophozoite B has a slit on the dorsal surface along the ventral groove. Sample was prepared in the same way as described in the legend for Fig. 3.

Trophozoite adhered to the cover glass by means of the VLF (Fig.3). Many trophozoites had microvillus-like projections protruding from the edge of the VLF, especially the posterior region (Fig. 5), as described by Feely et al. (1984).

3.2.2 Morphometric assay

To study the size variation among 8 isolates, the dimensions of trophozoites were measured (Table 4). The average length of trophozoites from 8 isolates ranged from 13.6 to 17.6 μm, and the average width ranged from 7.2 to 8.2 μm. Standard deviation of the measurement of each isolate was less than 11% of the average, indicating that the size variation in one culture was not large. The sizes of our isolates were larger than those of the isolates from small rodents in southern Ontario which ranged from 13.4 to 15.5 μm in length and from 6.7 to 7.5 μm in width (Grant and Woo 1978b).

The ratio between length and width of the trophozoite body (L:W) was in the range of 1.8 to 2.2 (Table 4). Fig. 6 shows the distribution of L:W of 8 isolates used in our study. The smallest isolate, D3, was 20% shorter in length and 10% narrower in width compared with the largest isolate, B5. This distribution of L:W (Fig. 6) was wider than that of 3 G. duodenalis isolates obtained from small rodents and measured by Grant and Woo (Fig. 7).

3.3 DNA analysis

To study the genetic variation among 8 isolates, their DNA restriction fragment patterns were compared. DNA fragments observed in this study are considered to be derived from Giardia DNA, because

Figure 5 SEM photograph showing a trophozoite with microvillus-like projections. The arrow indicates microvillus-like projections along the lateral border of the ventrolateral flange. Sample was prepared in the same way as described in the legend for Fig. 3.

Table 4 Dimensions of trophozoites of G. duodenalis isolates

Isolates	Length(L;μm)*	Width(W;μm)*	L:W*
WB	16.4 ± 1.0	7.8 ± 0.6	2.1 ± 0.4
H7	15.2 ± 1.3	7.7 ± 0.8	2.0 ± 0.2
H8	14.4 ± 0.9	7.4 ± 0.6	2.0 ± 0.2
B5	17.6 ± 1.4	8.2 ± 0.8	2.2 ± 0.1
PB1	14.1 ± 1.1	7.3 ± 1.0	1.9 ± 0.2
MR4	15.4 ± 1.1	7.8 ± 0.6	2.0 ± 0.1
S1	13.8 ± 1.4	7.5 ± 0.8	1.8 + 0.2
D3	13.6 ± 1.1	7.2 ± 0.6	1.9 ± 0.3

* Average ± standard deviation

The length and width of the trophozoites were determined by measuring the first 40 intact cells encountered in the SEM photographs of each isolate. The unit bar (10 μm) automatically printed in the photograph was used as the unit length for measurement. The maximum width at the level of nuclei was defined as width. The maximum distance from the anterior end to the posterior end of the trophozoite body was derined as length.

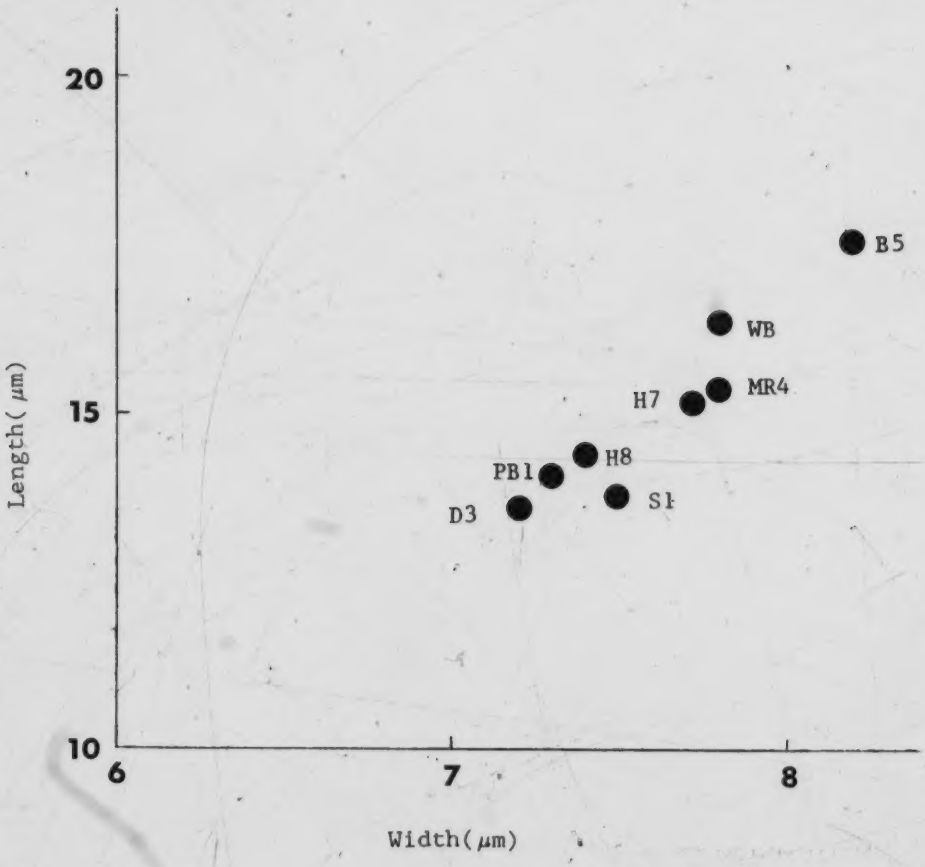


Figure 6 Graphical representations of the ratio between length (L) and width (W) of trophozoites of G. duodenalis isolates from various hosts in Southern Alberta. The values of L and W were drawn from Table 4.

Fig. 7 has been removed because of the unavailability of copyright permission.

Figure 7 Graphical representations of the ratio between length and width of G. duodenalis isolates from rodents in Southern Ontario. A) G. simoni, B) G. peromysci, C) G. microti. (Ref. Grant and Woo 1978b).

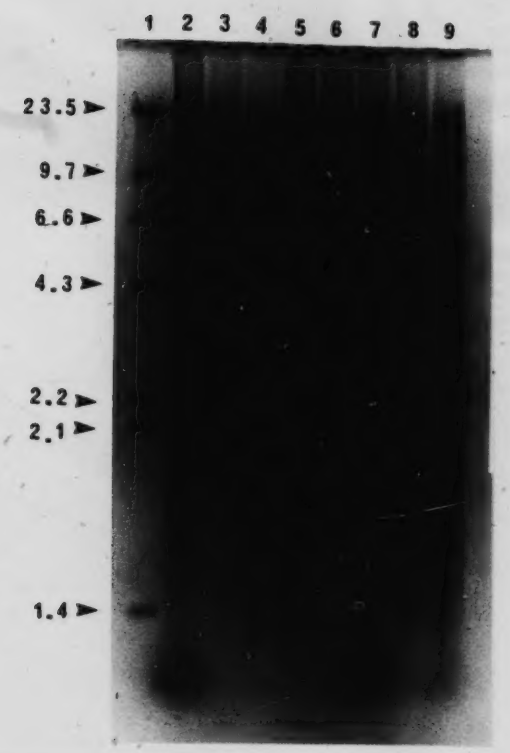
no bacterial contamination was detected in the Giardia cultures used for DNA isolation.

For DNA analysis, DNA is first digested by restriction endonucleases. This digestion of genomic DNA generates a unique set of different sized DNA fragments depending on the base sequences, because each endonuclease cuts DNA at a site of specific sequences. The size distribution of these fragments is unique to the genotype and can be analyzed by agarose gel electrophoresis. Ethidium bromide staining of this gel allows the visualization of DNA fragments. The multiple copies of repetitive DNA sequences are visualized as distinct bands while single-copy DNA cannot be visualized because of low DNA concentration present in a single-copy sequence (Curran et al. 1985). These distinct bands can be diagnostic characters to identify species of parasites.

In our study, ethidium bromide staining of DNA, digested with BamHI, HindIII, or PstI, did not show any difference in fragment patterns among isolates (Fig 8, 9, and 10). BamHI digestion resulted in 3 common major bands, 1.6, 2.0, and 2.5 kb in size, respectively (Fig. 8). HindIII digestion demonstrated one major common band of 4.0 kb (Fig. 9). PstI digestion also gave the same size of major band common among isolates (Fig. 10).

Theoretically, DNA-DNA hybridization allows the detection of more subtle differences, since only those DNA fragments that have compatible sequence with a probe can be visualized. However, in our study DNA hybridization did not reveal any difference among isolates. DNA digested with BamHI and hybridized with WB probe showed 3 major common bands, 1.6, 2.0, and 2.5 kb in size (Fig. 11, A). Hybridization with H7 or D3 probe showed fragment patterns almost identical with those

Figure 8 Agarose gel electrophoresis of BamHI-restricted DNA from 8 G. duodenalis isolates stained with ethidium bromide. DNA was isolated from a one-litre culture of each Giardia isolate by extracting DNA from the cell lysate. Contaminating proteins, RNA and other cell components in the DNA extract were removed with a mixture of phenol, chloroform and iso-amyl alcohol. DNA was partially purified by ethanol precipitation and dialysis against 10 mM Tris-HCl buffer (pH 7.5). Approximately 1 μg of DNA per sample was digested with 2 to 5 units of BamHI and electrophoresed overnight at 30V/cm in a 1.0% agarose gel. DNA fragments in the gel were visualized by ethidium bromide staining. Lane 1, molecular size standards (HindIII-digested lambda phage DNA); lane 2, WB; lane 3, H7; lane 4, H8; Lane 5, B5; Lane 6, PB1; lane 7, MR4; lane 8, S1; lane 9, D3.


Bam HI
1 2 3 4 5 6 7 8 9
23.5
9.7
6.6
4.3
2.2
2.1
1.4

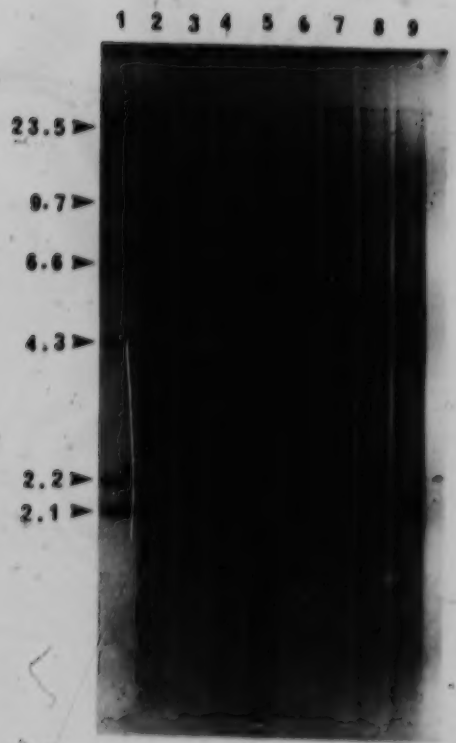


Figure 9 Agarose gel electrophoresis of HindIII-restricted DNA from 8 G. duodenalis isolates stained with ethidium bromide. Lanes are the same as in Fig. 8. DNA samples were processed in the same way as described in the legend for Fig. 8, except that HindIII was used instead of BamHI.

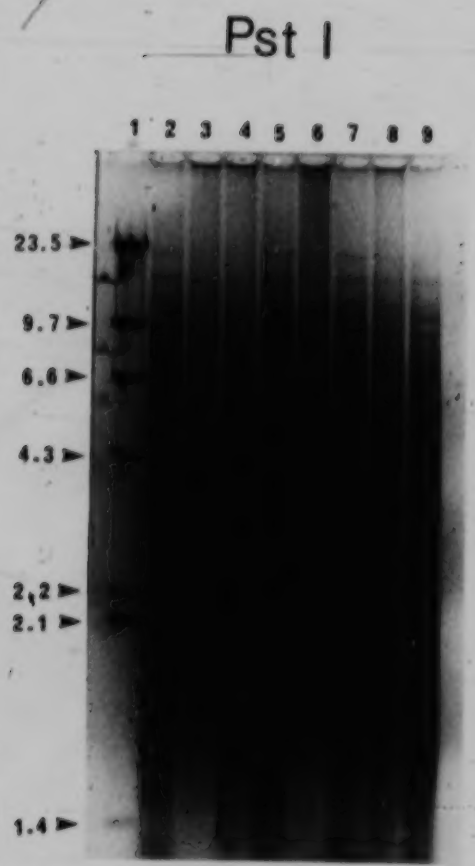


Figure 10 Agarose gel electrophoresis of PstI-restricted DNA from 8 G. duodenalis isolates stained with ethidium bromide. Lanes are the same as in Fig. 8. DNA samples were processed in the same way as described in the legend for Fig. 8, except that PstI was used instead of BamHI.

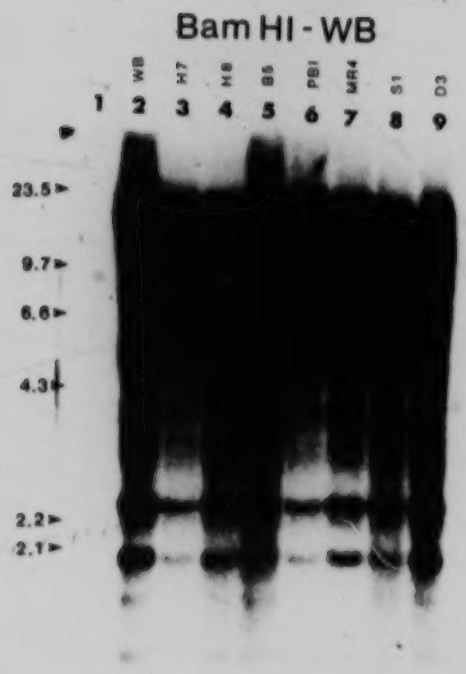
Figure 11 Autoradiograph of a Southern blot of BamHI-restricted DNA from 8 G. duodenalis isolates hybridized with 3 different ^{32}P-labeled DNA probes. DNA was digested with BamHI and electrophoresed in an agarose gel as described in the legend for Fig. 8. DNA in the gel was transferred to nitocellulose filter electrophoretically, and hybridized with WB, H7 or D3 DNA probe under stringent conditions in 50% formamide at 37°C overnight using 10^6 - 10^7 CPM of probe. After washing in 2xSSC with 0.2% SDS at room temperature and at 65°C, and in 0.2xSSC at 65°C, the blot was exposed to Kodak X-Omat AR film with two intensifying screens at -70°C overnight to autoradiograph. Lanes are the same as in Fig. 8.

(A) Hybridization with WB human isolate DNA probe.

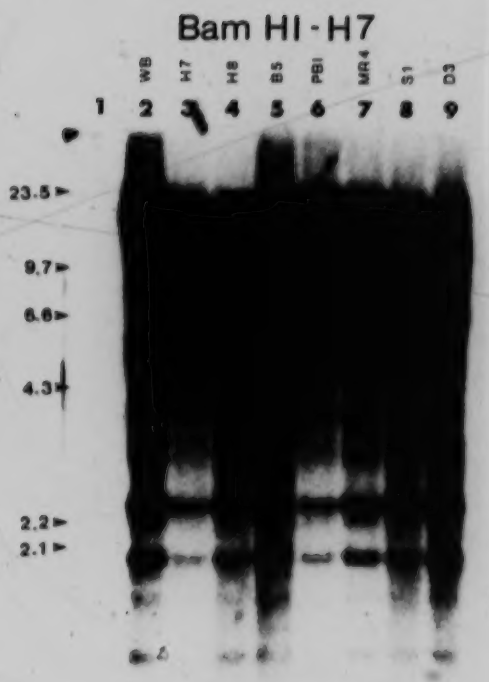
(B) Hybridization with H7 human isolate DNA probe.

(C) Hybridization with D3 dog isolate DNA probe.

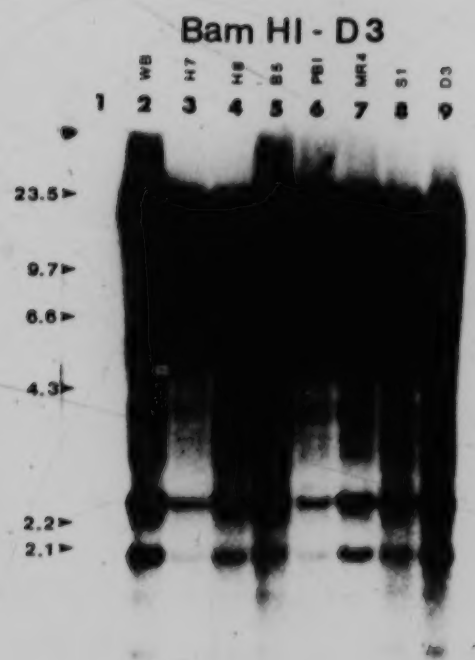
(A)


Bam HI - WB
1 2 3 4 5 6 7 8 9
WB H7 H8 B5 PBI MR4 S1 D3
23.5
9.7
6.6
4.3
2.2
2.1

(B)


Bam HI - H7
1 2 3 4 5 6 7 8 9
WB H7 H8 B5 PBI MR4 S1 D3
23.5
9.7
6.6
4.3
2.2
2.1

(C)



revealed by WB probe (Fig. 11, B and C). Likewise HindIII-digested DNA showed one major common band, 4.0 kb in size, hybridized with WB probe (Fig. 12, A). H7 and D3 probes gave the same fragment patterns (Fig. 12, B and C). Hybridized with WB probe, PstI-digested DNA showed one major band, 4.0 kb in size, which was common among all isolates (Fig. 13, A). Both H7 and D3 probes gave the same fragment pattern as WB probe (Fig. 13, B and C).

The sizes of major bands visualized by hybridization were identical with those of major bands visualized by ethidium bromide staining of electrophoresed DNA fragments.

3.4 Isozyme analysis

3.4.1 Electrophoretic mobility

The genetic variation among Giardia isolates was studied further by comparing the zymodemes, the electrophoretic mobility of isozymes. Isozymes are multiple molecular forms of a specific enzyme occurring either in a single individual or in different members of the same species. Members of a set of isozymes are very similar but have different enzyme properties in some ways. For example they may catalyze the same reaction but differ in their kinetics. This isozyme multiplicity can be caused by difference in gene sequence (Rider & Taylor 1980). Variations in isozymes can be considered as a direct reflection of genetic differences. Therefore, each isozyme subunit type can be used as a marker of its own gene.

In our study, glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), glucose phosphate isomerase (GPI), and malate dehydrogenase (MDH) were analyzed, and no electrophoretic heterogeneity was observed

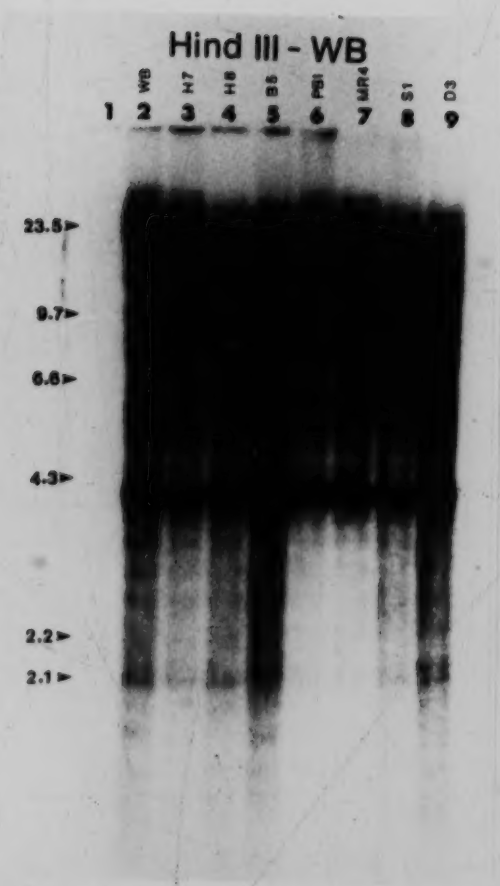
Figure 12 Autoradiograph of a Southern blot of HindIII-restricted DNA from 8 G. duodenalis isolates hybridized with 3 different ^{32}P-labeled DNA probes. DNA samples were processed in the same way as described in the legend of Fig. 11 except that DNA was digested with HindIII instead of BamHI. Lanes are the same as in Fig. 8.

(A) Hybridization with WB human isolate DNA probe.

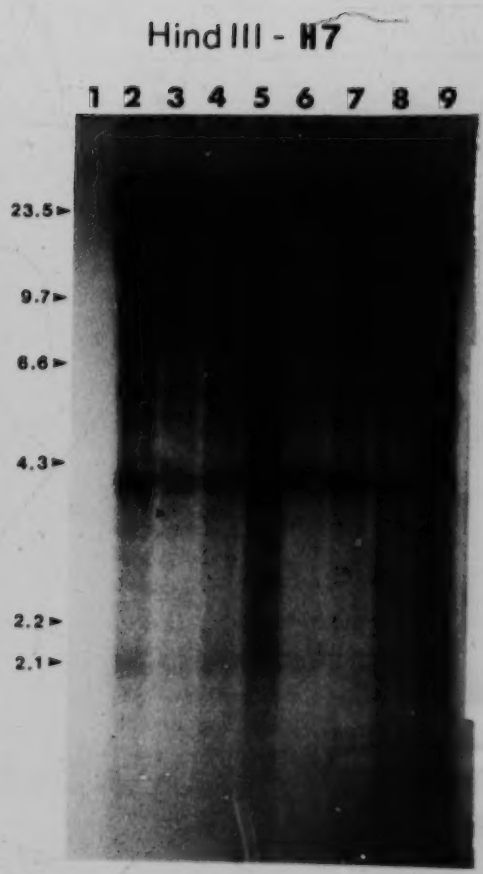
(B) Hybridization with H7 human isolate DNA probe.

(C) Hybridization with D3 dog isolate DNA probe.

(A)


Hind III - WB
WB
H7
H8
B5
PBI
MR4
S1
D3
1 2 3 4 5 6 7 8 9
23.5
9.7
6.6
4.3
2.2
2.1

(B)


Hind III - H7
1 2 3 4 5 6 7 8 9
23.5
9.7
6.6
4.3
2.2
2.1

(C)

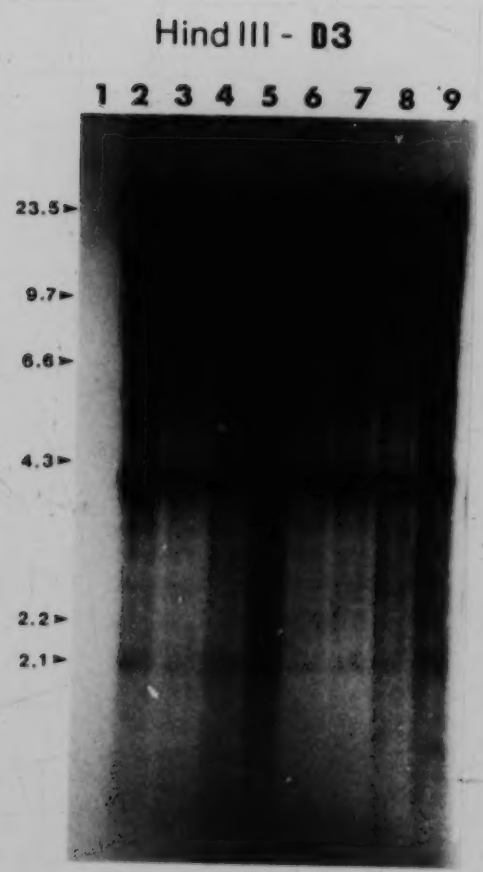
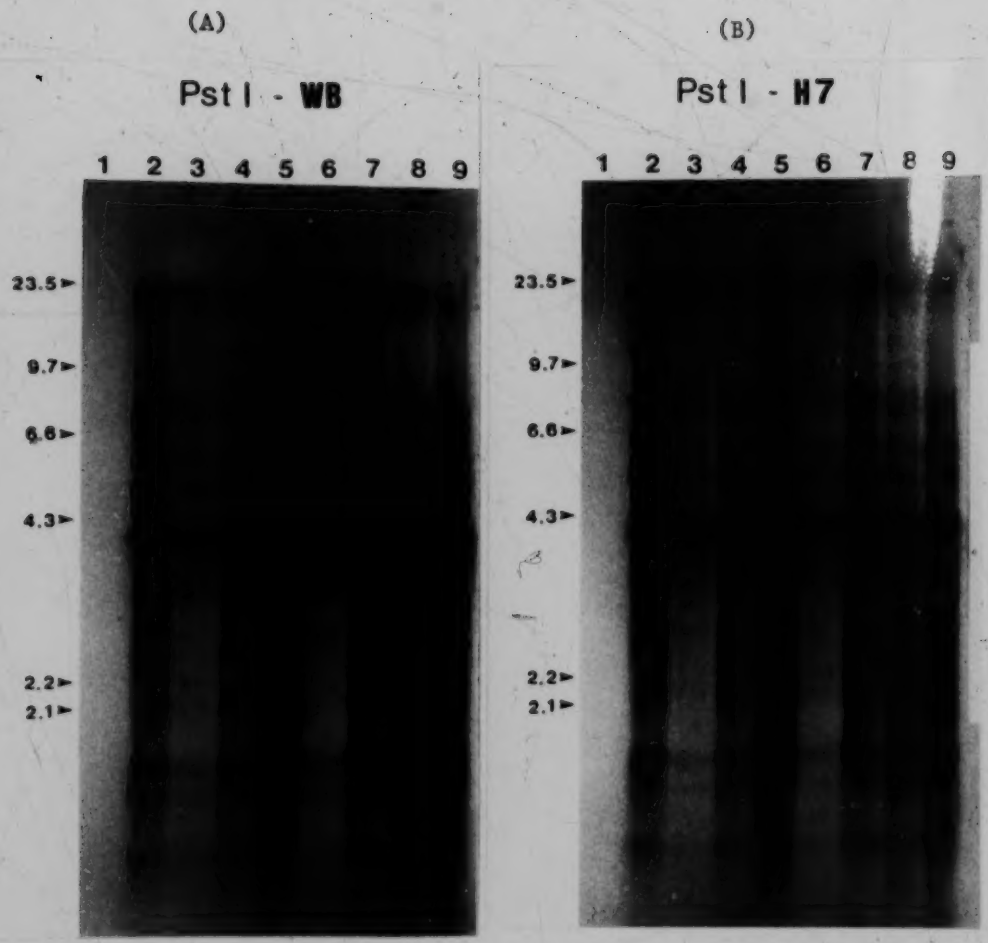


Figure 13 Autoradiograph of a Southern blot of PstI-restricted DNA from 8 G. duodenalis isolates hybridized with 3 different ^{32}P-labeled DNA probes. DNA samples were processed in the same way as described in the legend of Fig. 11 except that DNA was digested with PstI instead of BamHI. Lanes are the same as in Fig. 8.

(A) Hybridization with WB human isolate DNA probe.

(B) Hybridization with H7 human isolate DNA probe.

(C) Hybridization with D3 dog isolate DNA probe.


(A)
Pst I - WB
1 2 3 4 5 6 7 8 9
23.5
9.7
6.6
4.3
2.2
2.1
(B)
Pst I - H7
1 2 3 4 5 6 7 8 9
23.5
9.7
6.6
4.3
2.2
2.1

(C)

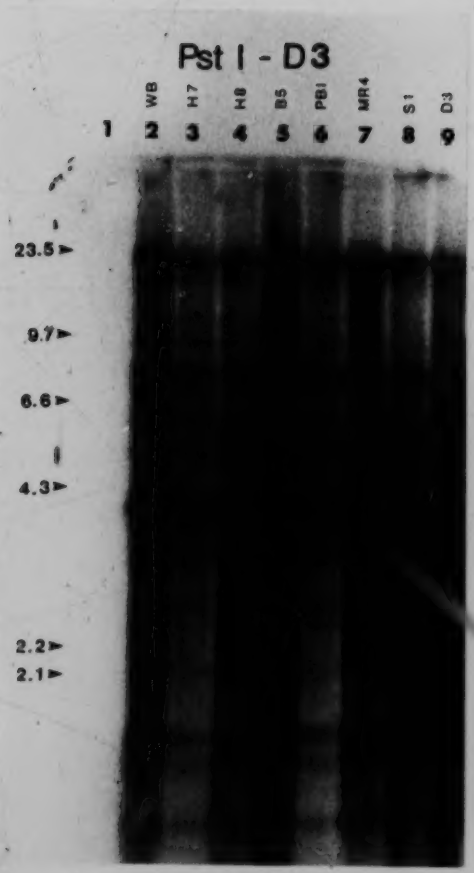
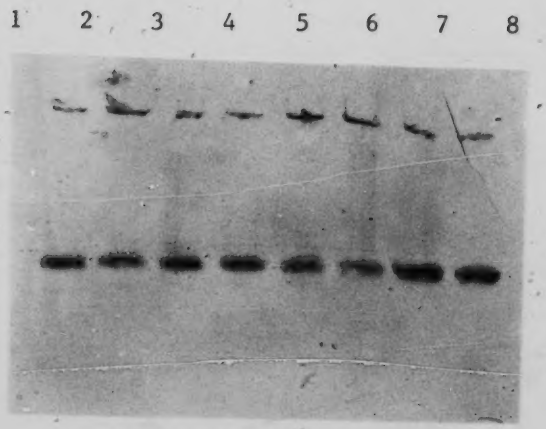


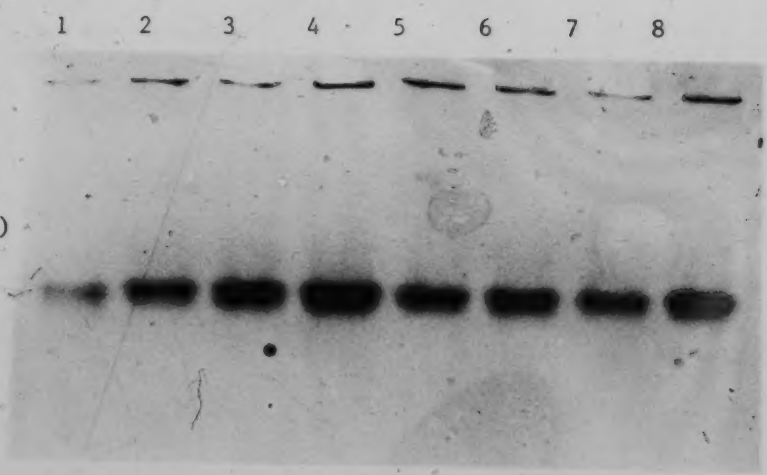
Figure 14 Zymodemes of 8 G. duodenalis isolates. Trophozoite lysates were prepared from each Giardia isolate by repetition of freezing and thawing, followed by centrifuging. Two to 4 μL of lysate was applied in 12.5% or 9% polyacrylamide gel and electrophoresed at 3 mA constant current overnight at 4°C. Gels and reservoir buffers were prepared according to the method of Laemnli (1970) except that sodium dodecyl sulfate was omitted. After electrophoresis, locations of ME, MDH and GPI were visualized by overlaying the developer reagents (Table 3) over the gels and incubating at 37°C in the dark until color was developed. To detect G6PD, electrophoresed proteins were transferred to a nitrocellulose filter by the method of Towbin et al. (1979), except that methanol was omitted from the transfer buffer. Constant current of 195 mA was applied at 4°C for 1 h. The enzyme was visualized by incubating the nitrocellulose filter with the developer reagent (Table 3) at 37°C in the dark for 20 min.

Lane 1, WB; lane 2, H7; lane 3, H8; lane 4, B5; lane 5, PB1; lane 6, MR4; lane 7, S1; lane 8, D3.

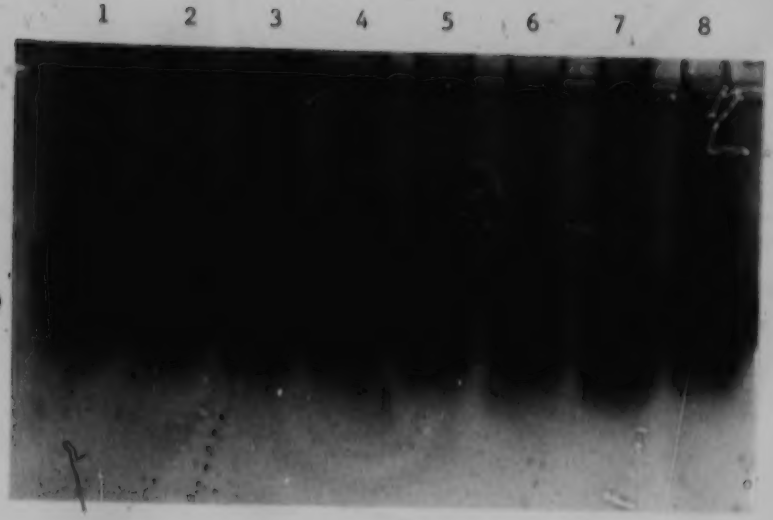
(A) G6PD in 9% PAGE, (B) ME in 12.5% PAGE, (C) GPI in 9% PAGE, (D) MDH in 12.5% PAGE.



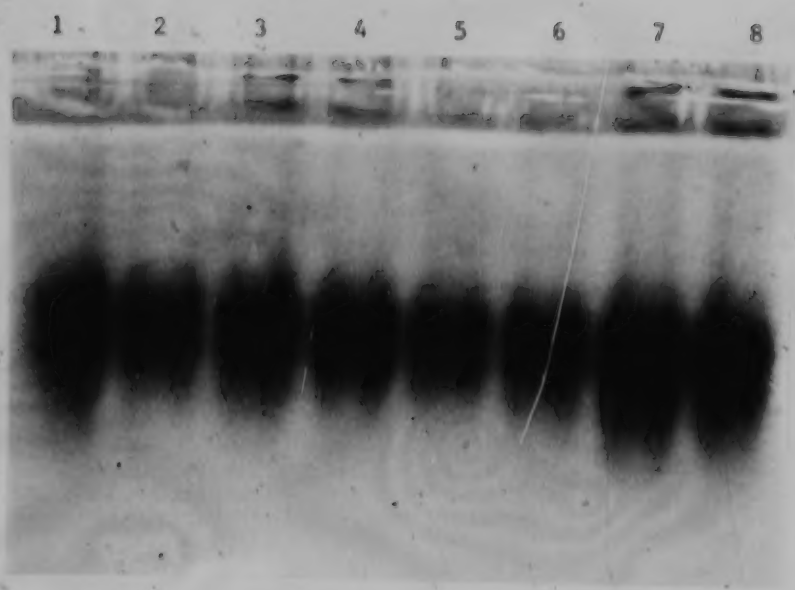
(A)



(B)



(C)



(D)

in any enzyme tested. Representative photographs displaying the migration patterns of isozymes are shown in Fig. 14. Multiple bands were not detected in any isozyme and results were consistent on repeated runs using cell lysates prepared on different occasions. The concentration of polyacrylamide gel affected the migration distance, but each enzyme from 8 isolates always migrated identically showing apparent electrophoretic homogeneity independent of the gel concentration.

3.4.2 Apparent Michaelis constants

Michaelis constants of G6PD and ME from 8 isolates were determined to study possible genetic variation. Apparent Km of G6PD and of ME are shown in Table 5 and 6. Apparent Km of G6PD from 8 isolates ranged from 194 to 346 μM. Apparent Km of ME ranged from 150 to 276 μM. The correlation between the reciprocal of substrate concentration and that of optical absorbency was high.

3.5 Intracellular pH

To study biological differences among Giardia isolates, the intracellular pH values were determined by the BCECF method. The red shift caused by Giardia cells was negligible (Fig. 15). The fluorescence of BCECF showed a linear relationship with pH in the range between pH 6.59 and pH 7.39 (Fig. 16). Intracellular pH of 8 isolates was 6.38 to 6.74 (Table 7).

Table 5 Apparent Michaelis constants of glucose-6-phosphate dehydrogenase from *G. duodenalis* isolates

Isolates	Apparent Km (μM)	Correlation coefficient of Lineweaver-Burk plot
WB	346	0.995
H7	242	0.988
H8	294	0.974
B5	248	0.992
PB1	278	0.987
MR4	210	0.976
SI	279	0.987
D3	194	0.992

The Km was determined by incubating 100 μL of trophozoite lysate, diluted 100 times with 100 mM Tris-HCl (pH 7.5), with the enzyme substrate mixture containing 100 μL of 300 mM $MgCl_2$ $6H_2O$, 0.2 mM NADP monosodium salt, 0.6 mL of 1% Triton X-100 in 100 mM Tris-HCl (pH 7.5), and 100 μL of substrate solution. A series of 5 doubling dilutions of glucose-6-phosphate starting from 2 mM was used as substrate solutions. After the incubation at 37°C for 30 min, the fluorescence produced by a reduced form of NADP was measured at 340 nm. A reaction mixture in which lysate was replaced with 100 mM Tris-HCl (pH 7.5) was used as a blank control. Triplicate tests were performed for each substrate dilution and the Km was obtained from a Lineweaver-Burk plot.

Table 6 Apparent Michaelis constants of malic enzyme from *G. duodenalis* isolates

Isolates	Apparent Km (μM)	Correlation coefficient of Lineweaver-Burk plot
WB	209	1.000
H7	276	0.999
H8	273	0.999
B5	178	0.999
PB1	150	1.000
MR4	173	0.999
S1	180	0.999
D3	228	0.999

The Km was determined by incubating 100 μL of trophozoite lysate, diluted 100 times with 100 mM Tris-HCl (pH 7.5), with the enzyme substrate mixture containing 100 μL of 6.6 mM $MnCl_2$, 100 μL of 3.3 mM of NADP monosodium salt, 0.6 mL of 1% Triton-100 in 100 mM Tris-HCl (pH 7.5), and 100 μL of substrate solution. A series of 5 doubling dilutions of malic acid starting from 0.833 mM, neutralized with NaOH, was used as substrate solutions. After the incubation at 37°C for 30 min, the fluorescence produced by a reduced form of NADP was measured at 340 nm. A reaction mixture in which lysate was replaced with 100 mM Tris-HCl (pH 7.5) was used as a blank control. Triplicate tests were performed for each substrate dilution and the Km was obtained from a Lineweaver-Burk plot.

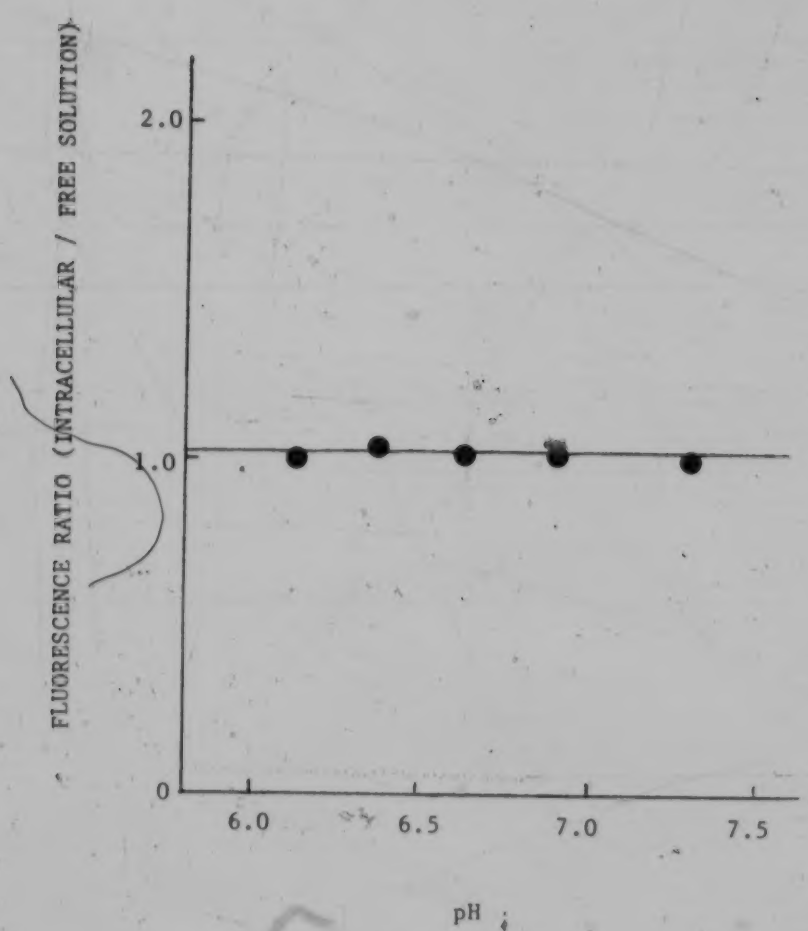


Figure 15 pH dependence of BCECF red shift. WB trophozoites were loaded with BCECF as described in Materials and Methods, and the fluorescence of BCECF trapped in cells and that of BCECF released in free solution were measured at the indicated pH.

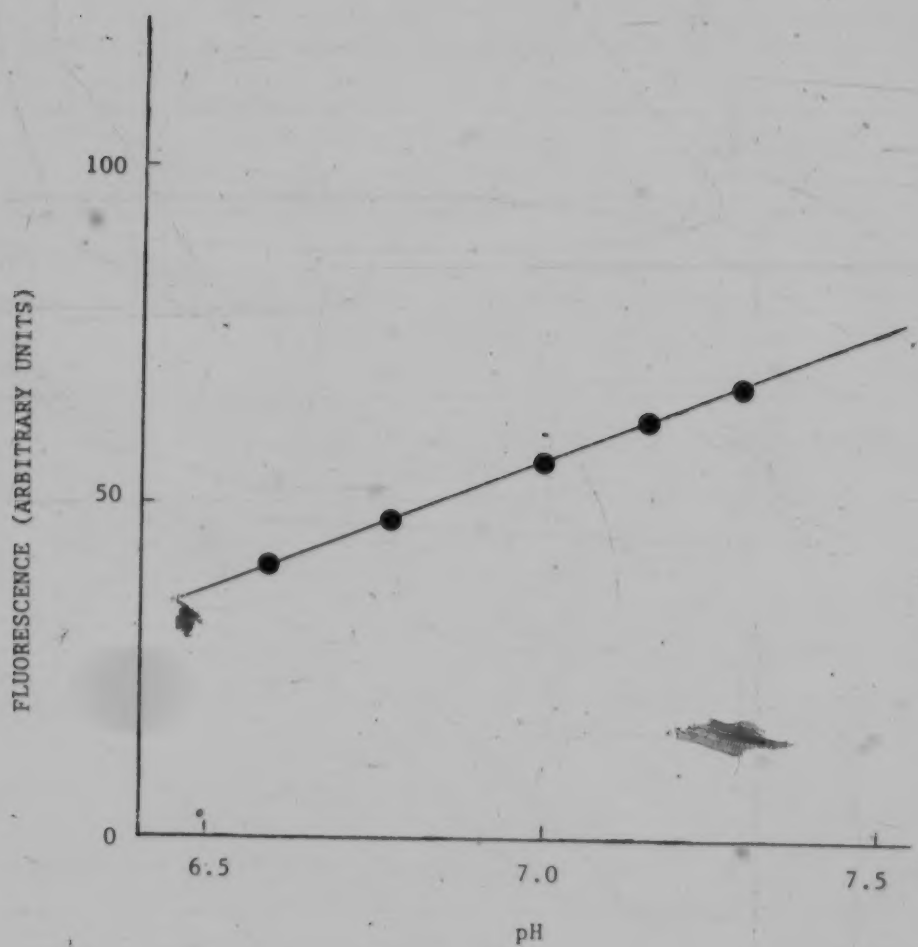


Figure 16 pH dependence of BCECF fluorescence. WB trophozoites loaded with BCECF were disrupted by Triton X-100. The fluorescence of the released BCECF was measured at the indicated pH by changing pH with HCl.

Table 7 Intracellular pH of G. duodenalis isolates

Isolate	pHi	Correlation coefficent* between pH and fluorescence
WB	6.55	0.984
H7	6.52	0.995
H8	6.54	0.994
B5	6.51	0.994
PB1	6.48	0.985
MR4	6.38	0.998
S1	6.64	0.998
D3	6.74	0.998

* Average of at least three measurements
pHi: intracellular pH

Intracellular pH was measured according to the method of Grinstein et al. (1984a). Freshly harvested trophozoites were resuspended to 10^8 cells/mL in 2 mL of choline$^+$ solution (pH7.2), consisting of 140 mM choline chloride, 10 mM glucose, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES, and loaded with bis (carboxy-ethyl) carboxy fluorescein acetoxymethyl ester (BCECF). After resuspending trophozoites in 1 mL of fresh choline$^+$ solution, the fluorescence of this cell suspension was measured at 37°C. The pH corresponding to the intensity of this fluorescence was defined as intracellular pH and determined from a calibration curve between pH and fluorescence. The calibration was carried out for each sample by disrupting the cells with 100 µL of 1% Triton X-100, followed by repetitive titration of the medium with 1N HCl. Intracellular pH was measured at least 3 times for each isolate and the average was calculated.

3.6 Agglutination assay

Trophozoites of 8 G. duodenalis isolates were reacted with ascites fluid containing anti-WB monoclonal antibody to test for the existence of a common antigen. Monoclonal ascites fluid caused agglutination of all isolates while control ascites fluid did not show any effect, indicating that the 8 isolates share a common antigen (Fig. 17). Agglutination started right after the addition of monoclonal ascites fluid. No cytolethal effect was observed for at least 2 h after the addition of anti-WB monoclonal antibody.

3.7 Indirect immunofluorescence microscopy

The existence of a common antigen was studied further with indirect immunofluorescence microscopy using anti-WB monoclonal antibody. All isolates showed fluorescence reacting with monoclonal ascites fluid, which indicates that the isolates studied share a common antigen. Control ascites did not show fluorescence. Table 8 shows the intensity of fluorescence observed with 8 isolates. Both body and flagella were stained (Fig. 18A). Most cells had fluorescence all over the body surface, but some cells showed fluorescence only at the surrounding edges of the bodies. This difference in staining pattern may be caused by uneven staining. Each isolate included cells showing these two types of fluorescence. Fluorescence on the flagella was observed only in the region near the body. The nuclear region was not stained (Fig. 18B).

Figure 17 Agglutination of 8 *G. duodenalis* isolates by anti-WB monoclonal antibody. Freshly harvested *Giardia* cells of each isolate were resuspended in PBS to 9×10^5 cells/mL, and 50 µL of each suspension was added in wells of a 96-well tissue culture plate. One µL of mouse ascites fluid containing the monoclonal antibody raised against WB isolate was added in each well, and the plate was shaken gently for 30 min at room temperature. As a control, 1 µL of ascites fluid raised against SP2/0 myeloma cells was added in a well containing WB trophozoites. The photographs were taken using phase contrast microscopy.

WB

H7

H8

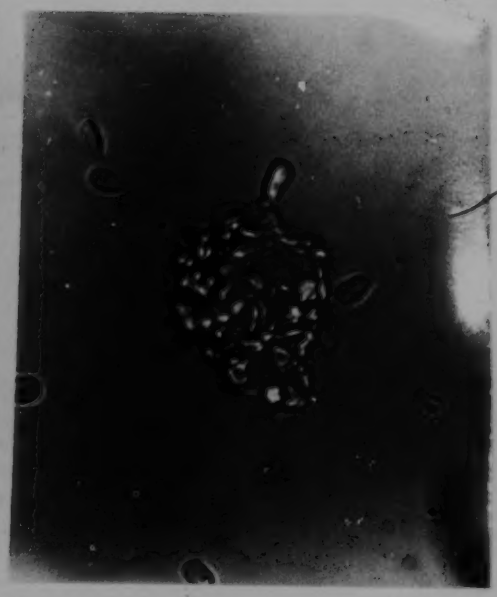
B5

PB1

MR4

S1

D3



CONTROL

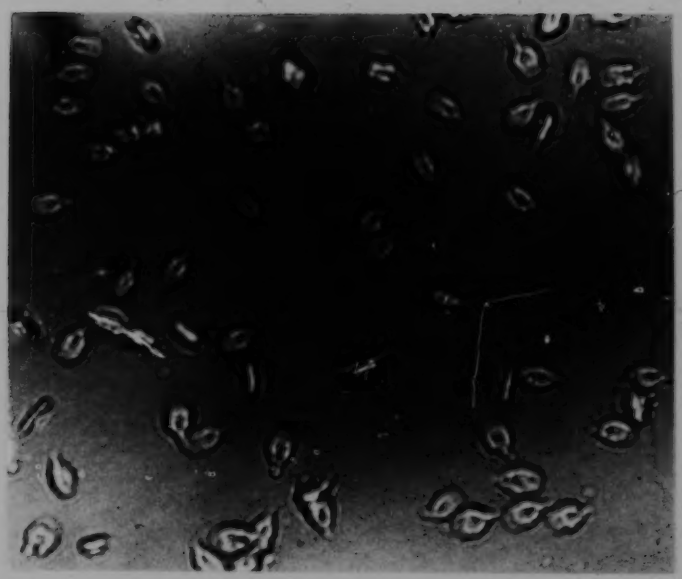


Table 8 Indirect immunofluorescence of G. duodenalis isolates with anti-WB monoclonal antibody

Isolates	McAb	Control
WB	++	-
H7	+	NA
H8	+	NA
B5	+	NA
PB1	++	NA
MR4	+	NA
S1	+	NA
D3	+	NA

McAb: Anti-WB monoclonal antibody
+: Intensity of fluorescence on a scale of + to ++.
-: No fluorescence.
NA: Not tested
Control is mouse ascites fluid produced by injecting SP 2/0 myeloma cells.

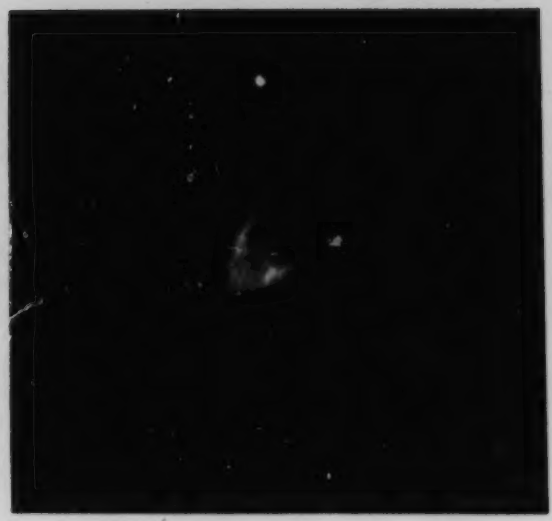
Figure 18 Indirect immunofluorescence of trophozoites of 8 G. duodenalis isolates with anti-WB monoclonal antibody. Trophozoites of 8 Giardia isolates, fixed in 0.37% formalin for 30 min and washed once in PBS, were applied on the gelatin-coated microscope slide and fixed in acetone. The ascites fluid containing anti-WB monoclonal antibody was diluted 1:100 in PBS, and applied on the fixed cells. Ascites control raised to SP2/0 myeloma cells was diluted in the same way and used as control. After incubation at 37°C for 30 min, the ascites fluids were washed off with PBS and the fluorescein isothiocyanate conjugated anti-mouse goat IgG, diluted 1:35 in PBS, was applied on the cells. This slide was incubated at 37°C for 30 min and, after rinsing in PBS, observed by fluorescence microscopy. Kodak Tri-X pan 400 film was used for photography.

(A) Trophozoite body and flagella were stained with anti-WB monoclonal antibody.

(B) Anti-WB monoclonal antibody did not stain nuclei.

(A)

(B)



3.8 Immunoprecipitation assay

The molecular weight of a common antigen recognized by the anti-WB monoclonal antibody was determined by reacting monoclonal ascites fluid with ^{125}I-labelled WB trophozoites. Monoclonal ascites fluid precipitated surface antigens of the WB isolate. The molecular weight of a major antigen was 105 kDa calibrated from molecular standards in 12.5% SDS-PAGE (Fig. 19, A1), or 99kDa calibrated from molecular standards in 7.0% SDS-PAGE (Fig. 19, B1). Table 9 shows the molecular weights of the antigens precipitated by monoclonal antibody. Control ascites fluid gave a dark spot at the bottom of the gel of 12.5% SDS-PAGE (Fig. 19, A2) but did not precipitate the 105-kDa or 99-kDa antigen in both 12.5% and 7.0% SDS-PAGE (Fig. 19, A2 and B2).

3.9 Western blot assay

Western blot assay was tried to determine the molecular weight of an antigen common among 8 isolates. Anti-WB monoclonal antibody in ascites fluid reacted with several antigens from WB isolate (Fig. 20, A1). These were denatured antigens electrophoresed in SDS-PAGE. Control ascites fluid did not react with antigens on the blot (Fig. 20, A2). Table 10 shows the molecular weights of antigens which reacted. The immunoprecipitation assay detected a major surface antigen of 105 kDa in 12.5% SDS-PAGE (Fig. 19A), but this antigen was not detected by Western blot assay (Fig. 20, A1).

Anti-WB monoclonal antibody reacted stronger with non-denatured WB antigens (Fig. 20, B1) than with denatured antigens (Fig.20, A1), although the amount of antigens per lane was identical in the blots A1 and B1 in Fig. 20. Control ascites fluid did not react with antigens on

Figure 19 Autoradiograph of immunoprecipitated WB trophozoite surface antigens. Live WB trophozoites were labeled with ^{125}I and sonicated. Solubilized antigens were reacted with mouse ascites fluid containing anti-WB monoclonal antibody, and precipitated with protein A-Sepharose. Mouse ascites fluid raised against SP2/0 myeloma cells was used as control. Immunoprecipitated antigens were solubilized with SDS, and after centrifuging, the supernatant was electrophoresed in 7.5% or 12% SDS-PAGE at a constant current of 40 mA for 2 h according to the method of Laemnli (1970). After electrophoresis, the gel was stained with Coomassie blue to visualize proteins and dried on filter paper under vacuum with heating. The dried gel was exposed to Kodak X-Omat AR film with one intensifying screen at -70°C for several hours to overnight to autoradiograph. The molecular weights of antigens were calibrated from molecular standards. The low molecular weight markers include phosphorylase b (94,000), bovine serum albumin (BSA; 67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400). The high molecular weight markers consist of myosin (200,000), β-galactosidase (116,250), phosphorylase b (95,500), BSA (66,200), and ovalbumin (45,000). Molecular weights are shown in parentheses.

Lane 1, Monoclonal antibody; lane 2, Control.

(A) 12.5% SDS-PAGE (B) 7.0% SDS-PAGE

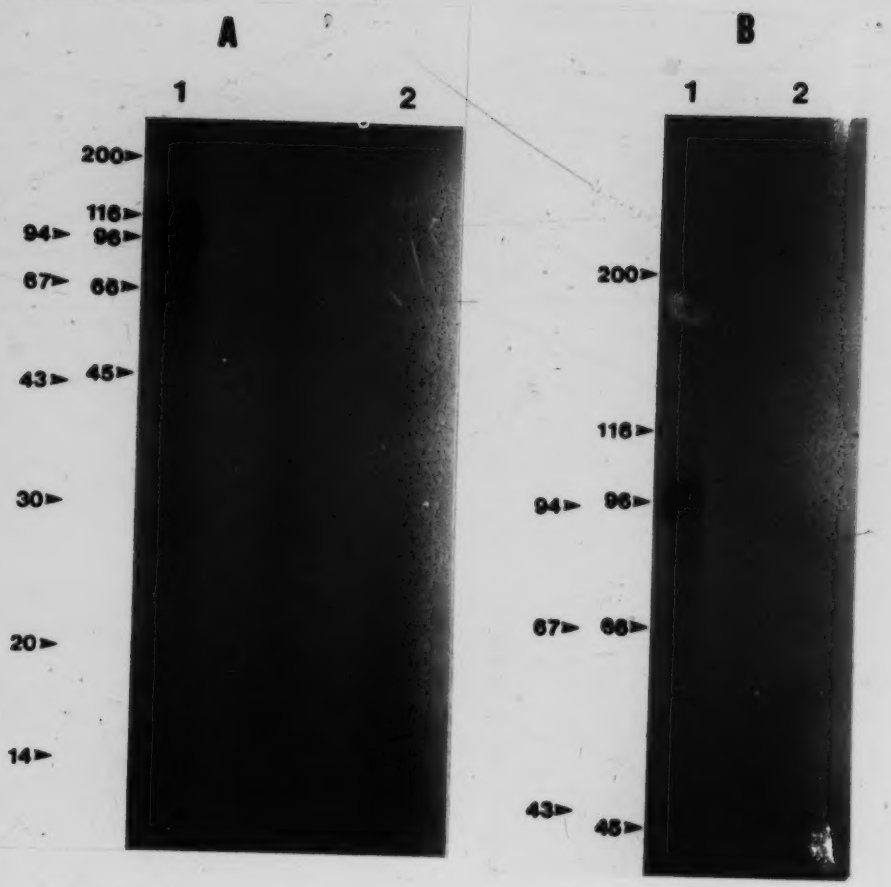

A
1
2
200
116
94
96
67
68
43
45
30
20
14
B
1
2
200
116
94
96
67
68
43
45

Table 9 Molecular weights of WB trophozoite surface antigens immunoprecipitated by anti-WB monoclonal antibody

Band	Molecular weight	Molecular weight[1)] of equibarent band on Western blot
1*	105,000	
2	91,000	94,000
3	66,000	64,000

Note) Molecular weights were calibrated from the migration distances of WB antigens and molecular standards in Fig. 19.
* major band
1) Data were drawn from Table 10.

Figure 20 Autoradiograph of Western immunoblots of WB trophozoites reacted with anti-WB monoclonal antibody. Sonicated Giardia WB trophozoites were electrophoresed in 12.5% SDS-PAGE at 40 mA for 2 h. In each lane 100 μg protein of sample was applied. After electrophoresis, protein in the gel was transferred to a nitrocellulose filter applying a constant current of 195 mA for 1 h. The blot was incubated in 2% ovalbumin in TSA (OA-TSA), then reacted with mouse ascites fluid containing anti-WB monoclonal antibody, diluted 1:100 in 2% OA-TSA, at room temperature overnight. Ascites fluid raised against SP2/0 myeloma cells was used as a control. The blot was washed in TSA, and reacted with 5 x 10^6 CPM of ^{125}I-labeled protein A in 200 mL of OA-TSA. After washing, the reacted blot was dried and exposed to Kodak X-Omat AR film with 2 intensifying screens at - 70°C to autoradiograph. The molecular weights of antigens were calibrated from the molecular weight markers including phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,400). A blot of undenatured antigens, prepared by electrophoresing WB antigens without SDS, was processed in the same way. Lane 1, Monoclonal antibody; Lane 2, Control. (A) Western blot with SDS, (B) Western blot without SDS

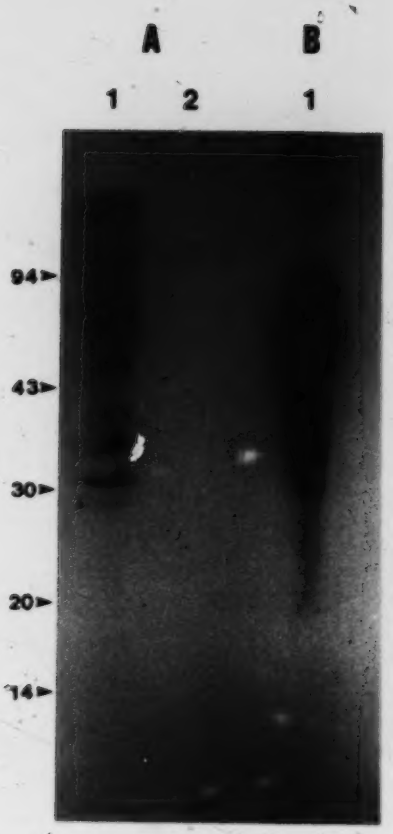

A
B
1
2
1
94
43
30
20
14

Table 10 Molecular weights of WB trophozoite antigens on a Western blot recognized by anti-WB monoclonal antibody

Band	Molecular weight
1*	185,000[1)]
2	94,000
3*	64,000
4	60,000
5	48,500
6	34,000
7	30,000

Note) Molecular weights were calibrated from the migration distances of WB antigens and molecular standards in Fig. 20A.
* major bands
1) Beyond the range of standards.

the blot of non-denatured WB antigens.

4. Discussion

4.1 Elimination of bacterial contamination

The original cultures of G. duodenalis isolates used in this study were contaminated with bacteria, probably Pseudomonas and Alcaligenes, as reported by Wallis and Wallis (1986). This bacterial contamination was successfully eliminated by incubating harvested trophozoites in 50 mM phosphate-buffered saline pH 7.0 (PBS) containing penicillin (1.0 mg/mL), gentamicin (1.0 mg/mL), and ticarcillin (2.5 mg/mL) and then using sterile conditions for subsequent growth of Giardia.

Our method is more simple and effective than the methods used by Wallis and Wallis (1986) to eliminate bacterial contamination from Giardia cultures. These workers tried several methods such as a nylon fiber column method or an adherence method, but their methods were only partially successful. Finally, they cleared the intestinal flora of the animals infected with Giardia by administrating gentamicin before collecting trophozoites. The trophozoites extracted from the intestine were cultured in medium containing gentamicin and ampicillin (200 μg/mL each), and the medium was changed twice a day initially and every 2 or 3 days later on. In this way, they could control bacterial overgrowth in their cultures, but did not succeed in eliminating the bacterial contamination completely.

In our study, the complete elimination of bacteria was confirmed by culturing the trophozoites in medium without antibiotics. The result showed no bacterial growth in Giardia cultures after 2 weeks of incubation. Therefore, there is little possibility that substances from contaminating bacteria, such as bacterial DNA or enzymes, would affect

the results of experiments performed in this study. Our method for the elimination of bacteria from Giardia cultures will be applicable to other Giardia isolates whose bacterial contamination causes serious problems in biochemical characterization.

4.2. Morphological diversity

Based on general morphology and capability of being cultured in TYI-S-33 medium, all isolates collected in Southern Alberta were of the G. duodenalis type and indistinguishable from each other.

The trophozoites were all teardrop-shaped, typical of the G. duodenalis type or the G. psittaci type, which is a new species reported by Erlandsen and Bemrick (1987). The dimensions of our isolates were from 13.6 to 17.6 μm in length and from 7.2 to 8.2 μm in width (Table 4). Feely et al. (1984) reported that G. agilis trophozoites measure from 20 to 30 μm in length and from 4 to 5 μm in width, G. duodenalis trophozoites measure from 12 to 15 μm in length and from 6 to 8 μm in width, and G. muris trophozoites measure from 9 to 12 μm in length and from 5 to 7 μm in width. Therefore the isolates used in our study were of the G. duodenalis type which is slightly longer than the G. muris type and shorter and much wider than the G. agilis type.

The presence of a ventrolateral flange surrounding the trophozoite body (Fig. 3) confirmed that the isolates used in this study were of the G. duodenalis type, not of the G. psittaci type which is morphologically distinguishable from G. duodenalis by the lack of a ventrolateral flange in the anterial region (Erlandsen & Bemrick 1987). Moreover, the ability to grow in TYI-S-33 medium indicated that the isolates used in

our study are the G. duodenalis trophozoites. Among 3 major groups of Giardia; G. duodenalis, G. muris and G. agilis, only G. duodenalis has been cultured in vitro to date using this medium (Meyer & Radulescu 1984).

The morphological difference among our 8 isolates was analyzed further according to the method of Grant and Woo (1978b). Some variation in size among them was detected (Table 4, Fig. 6), but it is not known whether this difference is significant enough to consider that the 8 isolates include different Giardia species. Grant and Woo (1978a) showed that the 3 G. duodenalis isolates used in their study were actually different species having different host specificities by animal cross-transmission experiments. Although the variation in size of their samples was smaller than that of our samples as shown in Fig. 6 and 7, their isolates were different species. This result suggests that the isolates used in our study, showing a larger variation in size than the samples studied by Grant and Woo (1978b), may include different species.

On the other hand, mongolian gerbils were used to excyst the original cysts of all isolates used in our study except WB and PB1. This means that gerbils served as a common host for the locally collected 6 isolates which have different original hosts, therefore their host specificities may be overlapping. To confirm this, other experiments such as cross-transmission between different animal hosts are needed.

Since few reports have been published on morphometric comparison of Giardia isolates obtained in a single geographic area, our finding may be valuable to understand the size variation of the G. duodenalis

type isolates from various animal hosts in a small area.

4.3 Genetic diversity

4.3.1 DNA

The restriction endonuclease digestion of genomic DNA generates various sizes of DNA fragments depending on the base sequence (Curran et al. 1985). These DNA fragments can be separated by agarose gel electrophoresis and repetitive DNA can be visualized as distinct bands with ethidium bromide staining (Curran et al. 1985). The subsequent hybridization of DNA fragments with an appropriate DNA probe is often used to characterize the species of parasitic protozoa such as Leishmania (Beverly et al. 1987) or Trypanosoma (Majiwa et al. 1985).

In our study, first the DNA fragment patterns were compared between Giardia isolates after ethidium bromide staining. The DNA from 8 isolates, digested with BamHI, HindIII or PstI, did not show any difference among them in DNA fragment patterns (Fig. 8, 9, and 10). Several distinct bands were observed in each isolate. These bands, which are the reflection of repetitive DNA, are not unusual for the genomic DNA of protozoa, or even nematodes which have larger DNA than protozoa. Similar distinct bands have been reported in Trypanosoma and nematodes.

Curran et al. (1985) showed from 3 to 13 of prominent DNA bands in various nematode isolates including Trichinella and Caenorhabditis. Majiwa et al. (1985) reported several distinct DNA fragment bands from Trypanosoma clones indicating the presence of repetitive sequences. Therefore, the distinct bands observed in our Giardia DNA samples were understood to be repetitive sequences and the meaning of these sequences

was not studied further in this project.

Next, Giardia DNA fragments were hybridized with DNA probes to study the degree of DNA sequence homology. Hybridization of Giardia DNA fragments with probes prepared from Giardia genomic DNA did not show any difference between 8 isolates (Fig. 10, 11, and 12). All possible combinations of 3 restriction enzymes (BamHI, HindIII and PstI) and 3 types of probes (two human isolates and one dog isolate) were tried, but the results were the same. This finding indicates the low genetic diversity among the G. duodenalis isolates used in our study.

It is not known how much difference in DNA sequence we can expect between Giardia species because there is no published paper on this topic. However it may be possible to estimate roughly the degree of genetic diversity among Giardia species based on the studies on Trypanosoma which, like Giardia, is a flagellated protozoan parasitizing vertebrates.

Majiwa et al. (1985) demonstrated that the difference in DNA fragment patterns among 13 Trypanosoma congolense clones can be revealed by ethidium bromide staining of DNA restricted with endonucleases. To digest DNA, they used Sau3A1, AluI, MboI, RsaI or HpaII, and all 5 enzymes revealed differences in DNA fragment patterns between isolates.

T. congolense and G. duodenalis are both closely related parasitic protozoa classified into the same subclass Zoomastigia (Kudo 1966), and they have a similar genome size. The genome size of Giardia is 3.1 X 10^7 bp (Nash et al. 1985) or 8 X 10^7 bp (Boothroyd et al. 1987) and that of Trypanosoma is 4.3 X 10^7 bp (Borst et al. 1982). Since the reported genome size of Giardia ranges from 3.1 X 10^7 to 8 X 10^7 bp, and that of Trypanosoma (4.3 X 10^7 bp) falls in this range, we can

assume that the difference in genome size between Giardia and Trypanosoma is small. The fact, that T. congolense shows genetic difference in DNA fragment patterns after endonuclease digestion while G. duodenalis does not, indicates that the genetic diversity among G. duodenalis isolates is much smaller than that of T. congolense isolates, although the sizes of their genomes are similar.

This is understandable when we compare the selective pressures which have acted in the courses of evolution of Trypanosoma and Giardia. T. congolense requires both insect and vertebrate hosts to complete its life cycle (Olsen 1974). As a contact between an insect host and a vertebrate host is essential, the habitat of T. congolense is ecologically limited to a specific area. This geographical isolation will allow genetic difference among T. congolense species living in different areas after a long period of evolution. In contrast, Giardia has a direct host cycle. They can live anywhere as long as hosts are available, so Giardia has little possibility of genetic diversity caused by geographic isolation.

In addition, genetic homogeneity will be maintained after reproduction of Giardia, since it reproduces only by binary fission (Marquardt & Demaree 1985). T. congolense, which has a nonobligatory sexual cycle, can reproduce sexually (Tait 1985). Sexual reproduction allows genetic exchange by recombination and increases the occurrence of genetic diversity. Therefore it is likely that Giardia will show less genetic diversity than Trypanosoma.

We may also be able to estimate the degree of genetic variation among Giardia isolates used in our study based on that of Leishmania, a flagellated protozoan like Giardia. Beverly et al. (1987) analyzed the

genetic diversity of 28 Leishmania isolates, and estimated the DNA sequence divergence among the major lineages of Leishmania isolates to be 13 to 25%; within species, 6 to 2%; and within subspecies, less than 0.5%.

DNA fragment patterns of Leishmania vary greatly between species and the difference can be revealed by the ethidium bromide staining of endonuclease-digested DNA (Beverly et al. 1987). The differences in DNA fragment patterns among G. duodenalis isolates are, however, not revealed using the same method (Nash et al. 1985, Fig. 8, 9, and 10 in our study). From this difference we can expect the genetic diversity within G. duodenalis isolates used in our study to be less than that of Leishmania species, that is, 6%.

The DNA hybridization technique should be a useful tool for the identification of Giardia species because it can detect genetic difference. However, there is only one report (Nash et al. 1985) applying this technique to the classification of Giardia isolates, and they analyzed isolates from a variety of geographic locations. Therefore our study may be valuable as the first application of DNA-DNA hybridization technique to the differentiation of Giardia isolates from a single geographic area.

4.3.2 Isozymes

Electrophoretic isozyme analysis has been used extensively for the differentiation of parasitic protozoa such as Leishmania (Le Blancq et al. 1986) and Plasmodium (Carter 1978). This technique is suitable especially for the differentiation of G. duodenalis because it is an asexual organism. Asexual organisms tend to show low levels of

variation between individual stocks and so establish a clonal population structure (Tait 1985). Therefore, theoretically few zymodemes are found and an adequate description of the population can be made from different geographical areas or hosts (Tait 1985).

In our study, all 8 isolates showed identical zymodemes in regard to 4 enzymes: glucose-6-phosphate dehydrogenase (G6PD), malic enzyme (ME), malate dehydrogenase NAD^+, and glucose phosphate isomerase (Fig. 14). These results indicate that the 8 isolates have genetically very similar isozymes, in other words, the genetic variation between the 8 isolates used in our study is small. Although little difference in electrophoretic mobility was observed in each of the enzymes tested, they are called "isozymes" in this paper because several authors (Korman et al. 1986, Baveja et al. 1986, and Meloni et al. 1988) have reported the isozymes of these enzymes.

Our finding disagrees with the results of previous studies reported by other authors. Thus far, three articles have been published on the analysis of isozymes from G. duodenalis isolates obtained in single geographic areas. All of them demonstrated significant heterogeneity among the isolates (Korman et al. 1986, Baveja et al. 1986, and Meloni et al. 1988)

This disagreement in findings may be due to the difference in methodology, especially the type of supporting matrix for electrophoresis, because we used polyacrylamide gel while starch gel was used in the above-mentioned articles. However, theoretically both gels should have the same fractionation capacity. Starch gel was used in our study initially, but enzyme bands diffused so widely in the gel during the incubation period with color developing reagents that it was

impractical to compare the difference in migration patterns. Therefore, polyacrylamide gel was used in our study instead of starch gel.

Traditionally starch gel has been used as the supporting matrix for isozyme analysis because workers have the most experience with this system (Harris 1976). In both starch gel and polyacrylamide gel, proteins are separated by the difference in charge and by the sieving effect of the gel matrix. While polyacrylamide gel has almost no charged groups, starch gel contains a small proportion of carboxyl groups, and this causes electroendosmosis toward the cathode (Gordon 1969). As a result, the bands formed by the larger proteins in polyacrylamide gel are usually considerably sharper than in starch gel (Gordon 1969). Actually, Betram et al. (1983) obtained sharper bands of Giardia isozymes, including G6PD and ME, by polyacrylamide gel electrophoresis and divided 5 G. duodenalis isolates into 3 groups.

Therefore, the type of gel, polyacrylamide or starch gel, used for the electrophoresis of isozymes would not significantly affect the zymodemes. If there were differences in isozymes that can be visualized by starch gel electrophoresis, polyacrylamide gel electrophoresis can also differentiate these isozymes. Our result of the identical zymodemes is considered to show the genetic homogeneity between WB and the 7 isolates obtained in southern Alberta.

In our study, the Michaelis constants (Kms) of G6PD and ME were determined. The results did not show significant difference in Kms between 8 isolates (Table 5 and 6). Km is the substrate concentration at which the observed velocity is half of the maximum reaction velocity. Km can be used as an indicator of differences between isozymes in affinity for their substrates (Moss 1982).

There is only one paper on Km analysis of Giardia isozymes. Betram et al. (1983) determined the Kms of G6PD, ME, and malate dehydrogenase NAD^+ from 3 G. duodenalis isolates using the same method as in our study except for the reaction temperature. The Kms of G6PD of their samples ranged from 360 to 691 μM, and those of ME ranged from 19.3 to 38.1 μM. Our results of Kms for G6PD were from 194 to 346 μM (Table 5) which are in the same order as their results, but Kms for ME ranged from 150 to 276 μM (Table 6) which are one order higher than their results. The higher Km indicates the slower reaction velocity or the weaker affinity for substrates, therefore it is likely that the higher Km values in our study may be caused by the high reaction temperature. Betram et al. (1983) incubated their samples at 30°C, while our samples were incubated at 37°C. The optimal temperature of malic enzyme from Giardia may be lower than 37°C, and at higher temperatures it may lose affinity for the substrate, malic acid.

4.4. Antigenic diversity

Agglutination assay, indirect immunofluorescence assay, and immunoprecipitation assay demonstrated a common antigen on the surface of all 8 isolates used in this study. The molecular weight of this antigen was 105 kDa according to the molecular weight of a major protein precipitated by the monoclonal antibody raised to Giardia WB isolate and electrophoresed by 12.5% sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 19A). It was calibrated more accurately to be 99 kDa by 7.0% SDS-PAGE (Fig. 19B).

The ascites fluid containing monoclonal antibody against WB caused agglutination of all isolates (Fig. 17), which indicates that they have a common surface antigen recognized by the monoclonal antibody. This result indicates that the 7 isolates found in southern Alberta are antigenically similar to WB and that they mae be closely related species which may infect different animal hosts.

The same monoclonal antibody stained the trophozoites of all isolates in indirect immunofluorescence assay (Fig. 18), which also supports the existence of an antigen common among the 8 isolates. Unger and Nash (1987) studied cross-reactivity of 11 different anti-Giardia rabbit sera with 8 Giardia isolates, and reported different staining patterns with indirect immunofluorescence among the isolates. Homologous sera stained only the surface of trophozoites while heterologous sera stained whole cells with diffused internal fluorescence. They interpreted this result as different antigens being located on the surface while common antigens were located inside of the cells.

The different staining patterns were observed in our experiment, too. However, the two types of staining, surface fluorescence and diffused internal fluorescence, were observed within each isolate. This difference in staining pattern may be caused by uneven staining. Another possibility is that the antigens recognized by our monoclonal antibody are distributed on only one side, ventral or dorsal surface. If the antigen were located only on the ventral surface and a trophozoite were attached to a microscopic slide with the ventral side up, it would show diffused fluorescence or total staining of the whole cell surface. If it lay with the dorsal surface up, only the edge would

show fluorescence, which can be described as surface fluorescence.

Several reports on the molecular weights of G. duodenalis surface proteins have been published (Einfeld & Stibbs 1984, Nash et al. 1983, and Clark & Holberton 1986). Compared with these articles, the common antigen demonstrated in our study seems to coincide with the 105-kDa protein determined by Einfeld and Stibbs (1984). Although some protein components of the Giardia cytoskeleton have been identified, including tubulin (Crossley and Holberton 1983), giardin (Crossley and Holberton 1983) and calmodulin (Munoz et al. 1987), these proteins are different from the common antigen demonstrated in our study because they are cytoskeleton components which are not exposed on the surface.

The monoclonal antibody used in our study precipitated multiple proteins, one major protein of 105 kDa and another 2 proteins, in the immunoprecipitation assay (Table 9, Fig. 19) and reacted with several bands on Western blot (Fig. 20A). This cross-reactivity of our monoclonal antibody suggests that it may not be monoclonal; however other workers also observed cross-reactivity with highly characterized monoclonal antibody, and this reaction has been reviewed by several authors (Lane & Koprowski 1982 and Nigg et al. 1982). For example, Torian et al. (1984) reported that their monoclonal antibody specific to Giardia flagella reacted with 7 Giardia protein bands on Western blot. Gallo and Anderson (1983) reported that monoclonal antibody raised against Chinese hamster brain microtubule proteins reacted with α-tubulin, β-tubulin, and several other polypeptides from brain extract in the Western blot analysis. Therefore we did not study further to test the monoclonality of our monoclonal antibody, assuming that the additional bands observed in the immunoprecipitation assay may

be proteolytic fragments or unrelated proteins with epitopes similar to a common antigen.

The control ascites fluid showed a dark spot on the film of the immunoprecipitation assay (Fig. 19, A2), indicating the existence of radioactive low molecular weight substance which was not detected in the monoclonal antibody reaction sample (Fig. 19, A1). This dark spot is most likely contaminating material because it is diffused without forming a specific band, therefore we did not study this substance further. Particularly because the purpose of this experiment was to determine the molecular weight of a surface antigen recognized by our monoclonal antibody, the low molecular weight substance observed in the control in Fig. 19 was not important and it was enough to know from this experiment that the control did not show the 105-kDa band.

The major band of 105 kDa, precipitated by monoclonal antibody in the immunoprecipitation assay, was not revealed on Western blot (Table 9, Fig. 20, A1). This result suggests that the epitope of this major antigen was denatured by sodium dodecyl sulfate during electrophoresis so that monoclonal antibody could not react.

Also, non-denatured antigens showed stronger reaction with monoclonal antibody (Fig. 20, B1) than denatured antigens (Fig. 20, A1) on Western blots. To prepare the two types of blots in Fig. 20 (denatured antigens in blot A1, and non-denatured antigens in blot B1), an identical volume of antigen solution was electrophoresed, so both blots should have the same amount of antigens. Therefore the difference in reaction indicates that sodium dodecyl sulfate destroys antigenicity and decreases the amount of antibody that can react with the antigens.

This finding suggests that some Giardia antigens naturally recognized by the host are denatured by sodium dodecyl sulfate and lose their antigenicity. Therefore the Western blot technique, commonly used to characterize antigens, may be unreliable for the determination of the molecular weights of antigens and the immunoprecipitation assay may be more suitable for this purpose.

4.5 Intracellular pH

There has been no report on the determination of the intracellular pH of Giardia and our experiment is the first application of a recently developed BCECF method to Giardia intracellular pH determination. The intracellular pH of the 8 G. duodenalis isolates were weakly acidic ranging from pH 6.3 to pH 6.8 (Table 7). Among them the intracellular pH of human isolates (WB, H7 and H8) fell in a small range from pH 6.52 to pH 6.55, showing biochemical similarity of these isolates.

The intracellular pH of Giardia may be related to the pH of their infecting sites, because it is only the duodenum, which has been traditionally considered to be the infecting site of Giardia (Olsen 1974), that has acidic pH in the vertebrate intestine (Croll 1973).

In some instances the environmental pH determines the specific intestinal segment invaded by a certain parasite (Von Brand 1979). It may be reasonable to think that Giardia acquired this acidic intracellular pH during the course of selective evolution or adaptation in one host.

Gillin and Reiner (1982) showed the attachment and survival of P1, a Giardia human isolate, was strongly affected by the pH of the medium with sharp optima at pH 6.85 to 7.0. At pH 7.8 the attachment rate was

26% and the survival rate was 4% of those at optimal pH 6.9. In the gastrointestinal tract, pH increases along the tract. The pH of the vertebrate duodenum is from 2.0 to 7.5; the upper small intestine, pH 7.7; the lower small intestine, pH 8.0; and the large intestine, pH 8.5 (Croll 1973). Therefore it is only the duodenum that has pH suitable for the survival and attachment of Giardia. Living in high pH environment, such as the upper or lower small intestine, is not beneficial for Giardia, so the acidic intracellular pH can be interpreted as indicating the pH of a natural infecting site, the duodenum.

Ward et al. (1987) studied the hemagglutinating activity of taglin, a trypsin-activated lectin from a Giardia human isolate, using rabbit erythrocytes. Taglin was maximally active within a narrow pH range. The optimum hemagglutinating activity was at pH 6.5 and a reduction of 1 pH unit resulted in abolition of activity. They discussed that this taglin activation could be involved in Giardia attachment to enterocytes and, therefore, the value of the optimum pH may be related to the pH of the infecting site. This optimum pH of 6.5 coincides with the intracellular pH of Giardia human isolates, from pH 6.52 to pH 6.55, determined in our study. Therefore it is likely that the intracellular pH of Giardia determined in our study may also be related to the pH of the infecting site of Giardia.

5. Conclusions

The 7 *Giardia* isolates obtained from various mammalian hosts in Southern Alberta and one *Giardia* human isolate of Afghanistan origin, WB, were compared in their general morphology, dimensions, DNA binding patterns, zymodemes, surface antigens and intracellular pH. The results showed that 7 isolates and WB were very similar in several aspects. They were morphologically indistinguishable from *G. duodenalis*. Their genetic diversity was low as demonstrated by DNA-DNA hybridization and isozyme electrophoresis. They shared a common surface antigen of 99 kDa, which was demonstrated by agglutination assay, indirect immunofluorescence assay, and immunoprecipitation assay, using anti-WB monoclonal antibody. Their intracellular pH were all weakly acidic ranging from pH 6.3 to 6.8.

These results indicate that the 7 isolates obtained in Southern Alberta are genetically and immunologically very similar to one another regardless of the species of their original hosts. Though they were also similar to WB of Afghanistan origin, the correlation between the geographic origin and the specificity of *Giardia* isolates may exist. This finding contrasts with previous studies reported by other authors which demonstrated the genetic or antigenic heterogeneity of *Giardia* isolates originating in small areas (Korman *et al.* 1986, Baveja *et al.* 1986, and Meloni *et al.* 1988).

Our finding suggests that the cross-transmission of *G. duodenalis* between different animals, including humans, living in a small area is likely to occur, and supports the hypothesis proposed by Erlandsen *et al.* (1988) that the cross-species transmission of *Giardia* may occur with

intermediate reservoirs of beaver and muskrat which are capable of infecting humans.

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